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CORRECTIONS.

On page 97, Vol. lxxii, No. 1, March, 1927, counting from the bottom of the page, line 7, read *multiplied* for *divided*; line 5, read *at 2°C.* for *used in the test*, line 4, read $Q \times A$ for $Q:A$.

THE DISTRIBUTION OF ELECTROLYTES IN DOGS FOLLOWING LIGATION OF BOTH URETERS.

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In the terminal stage of chronic nephritis there develops in the blood a variety of progressive chemical changes dependent, in part at least, upon impairment of kidney function. Among the most striking of these is the decrease in carbonate and chloride ions associated with increase in phosphate ions and non-protein nitrogen. These alterations are sufficiently large to serve as a clinical experiment capable of shedding light on the acid-base equilibrium, were it possible to study them satisfactorily. However, the patients are usually so ill that the other manifestations of their disease introduce a discouraging number of variables into an already complicated picture. In order to produce a simpler experiment, we have ligated the ureters in a series of dogs, and studied the composition of the blood prior to operation and shortly before death from renal insufficiency, the total period of observation extending over 3 to 4 days. During this time, the loss of electrolytes in stool and vomitus is shown, in most instances, to be relatively insignificant. If, in addition, the animals are given no food, the results obtained will disclose alterations which consist chiefly of an internal readjustment of the acid-base elements of the animals' blood and tissues. Experiments of this nature should, therefore, be subject to easier interpretation than those obtained in similar clinical studies.

EXPERIMENTAL.

Blood was drawn from the jugular vein without anesthesia. A few hours later both ureters were ligated (ether anesthesia) just below the kidney, through a median abdominal incision. At the

same time samples of skin and muscle were taken from Dogs VI, VIII, X, and XIII; and from Dogs X and XIII, a sample of brain tissue through a small trephined opening in the skull. After operation the dogs were placed in a metabolism cage, receiving no food and varying amounts of water (see Table III). In about 3 days the animals were very sick and were bled again. At autopsy, further samples of skin, muscle, and brain were obtained. In Dog IV the contents of the stomach and intestines were removed and analyzed for chloride and total base.

A 100 cc. Luer syringe containing 10 cc. of albolene was used for removing the 55 cc. of blood required for the examinations. 3 cc. were oxalated for hemoglobin, hematocrit, and whole blood phosphate determinations; 6 cc. were run under oil into an oxalate tube for whole blood CO_2 content and whole blood chloride; 2 cc. were run into two flasks for whole blood total base (see below); and the remainder was placed under oil in a narrow necked 50 cc. centrifuge tube, which was then covered with a rubber cap. This last sample was allowed to clot, centrifuged, and a sample removed for estimation of serum CO_2 content. The rest of the serum was pipetted off and used for phosphate, chloride, non-protein nitrogen, and, in some instances, sulfate determinations.

The hemoglobin determination was done on a modified Sahli apparatus (glass rod standards) standardized by oxygen capacity determinations. Hematocrits were done in duplicate, centrifuged to constant reading. It was found to be rather difficult to obtain satisfactory results from blood mixed with oil, so, with probably insignificant sacrifice of accuracy, we did not use samples oxalated under oil.

For whole blood total base, 8 cc. of distilled water were put in a 10 cc. volumetric flask and blood run in from the syringe up to the mark; the flask was then shaken until hemolysis was complete, when a 5 cc. aliquot was quickly removed for total base determination. The method employed is a modification of the Fiske method for total base in urine (1). The same method was applied to serum and to vomitus.

Chlorides in whole blood, serum, vomitus, and tissues were determined according to Van Slyke's method (2); CO_2 content by the method of Van Slyke and Neill (3). Total nitrogen was estimated by Kjeldahl (macro), non-protein nitrogen by Folin's

method (4), phosphates by the method of Fiske and Subbarow (5). Sulfates were determined by the gravimetric method of Loeb and Benedict (6).

Duplicate determinations were carried out on total base, total nitrogen, chloride, and hematocrit. Total base, chloride, carbonate, phosphate, protein anion, and sulfate are expressed in milli-equivalents per liter; non-protein nitrogen in mg. per 100 cc.

Base bound as carbonate was calculated from the CO_2 content by a formula derived from Hasselbalch's equation as follows:

$$\text{Antilog (pH - pK')} = \frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3}$$

$$\text{“ “ “ “} = \frac{\text{CO}_2 - \text{H}_2\text{CO}_3}{\text{H}_2\text{CO}_3}$$

$$\text{“ “ “ “} = \frac{\text{CO}_2}{\text{H}_2\text{CO}_3} - 1$$

$$\text{H}_2\text{CO}_3 = \frac{\text{CO}_2}{\text{Antilog (pH - pK')} + 1}$$

$$\text{BHCO}_3 = \text{CO}_2 - \frac{\text{CO}_2}{\text{Antilog (pH - pK')} + 1}$$

$$\text{B as BHCO}_3 \text{ (m-eq.)} = \frac{\text{CO}_2 - \frac{\text{CO}_2}{\text{Antilog (pH - pK')} + 1}}{2.24}$$

$$\text{“ “ “ “ at pH 7.35} = 0.423 \text{ CO}_2$$

(CO_2 expresses total content in volumes per cent.)

Base bound to phosphate was calculated according to L. J. Henderson (7), and base bound to protein according to Van Slyke, Wu, and McLean (8). A pH of 7.35 was assumed in all these calculations, an assumption which, of course, introduces a real inaccuracy, especially in the second specimen taken during undoubted acidosis. However, these inaccuracies are considerably less than the order of magnitude of the changes due to ligation; for example, the total acid in Dog VIII would be 1.8 m-eq. less if pH 7.1 were used. Another probable error is to be found in the

formula for base bound to protein, for this formula was derived from a study of pony serum. At pH 7.35 the factor for pony serum (8) is 1.78 P (P = protein per cent), while that for human blood is 2.03 P (9). We have arbitrarily used the former factor but it is quite possible that dog blood may approach nearer to human blood than to pony blood. If this be true, our figures for base bound to protein are appreciably too low.

The routine of the experiments was altered as the work progressed (Table III). Dog II was allowed water only and vomited several times. Dog III drank freely and vomited frequently, effecting a practical gastric lavage. Dog IV was given a little water on the 2nd day (by mistake) and vomited immediately afterward. At autopsy the colon was empty and only 22 cc. of thick mucoid material were recovered from the entire gastrointestinal tract; this was added to the vomitus before analysis. Dogs V and VII died before blood could be taken. Dog VI had no food or fluid, vomited practically nothing, and had no stools. Dog VIII was given 200 cc. of water on the afternoon of the 2nd day and 200 cc. more on the morning of the 3rd day. There was a little vomiting on the 3rd day.

Dog X lost more blood at operation than the other animals, due to difficulty in the removal of brain tissue. 200 cc. of water were given on the 2nd day and again on the 3rd. At autopsy the stomach was empty. Dog XIII lost much less blood during operation; on the 2nd day it was given 250 cc. of water and the same amount on the 3rd day: the latter was vomited at once. At autopsy, there were 300 to 500 cc. of fluid in the abdomen, which, unfortunately, was not analyzed; another unusual finding was retroperitoneal hemorrhage at the site of both ligations.

Dog IX was fasted (both water and food) on two occasions (Table II) for 3 day periods to observe the effect of fasting alone. Dog XIV had no alteration of diet or fluid intake, serving simply as a control of the effect of removing 55 cc. of blood.

DISCUSSION.

Table I groups together all the initial blood analyses. Included are thirteen apparently normal dogs (two observations on Dog IX) of varying weight, without, however, a constant foreperiod of

TABLE 1.
Normal Figures

Dog No	Date	Serum.										Whole blood.							Weight.	Skin.	Muscle	Brain.			
		Non-protein N	Protein	Total base	Cl	HCO ₃	PO ₄	Protein	Total acid.	B-A	Hb	Hematocrit	Total base	Cl	HCO ₃	PO ₄	Total acid.	B-A							
	1926	mg per 100 cc	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	per cent	vol per cent	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	kg	kg per sq dm	kg per sq dm	kg per sq dm			
II	Mar 22	40	7 1156	5 102	5 24	3	1 411	3 139	5 17	0	32	5 137	0 86	8 21	2	1 7108	9 28	1 12	7						
III	Apr. 12	22	6 8158	0 108	6 22	6	2 611	7 145	5 12	5	40	6 141	0 86	2 18	8	2 4107	4 33	6							
IV	" 26	29	6 6161	3 105	0 22	2	1 811	4 140	4 20	9	43	0 141	8 88	3 18	8	1 2108	3 33	5	9						
V	May 10	32	6 2168	3 104	9 25	9	2 810	8 144	4 23	9	44	7 157	8 79	5 20	4	2 4102	3 55	5	9	0					
VI	" 17	27	6 6155	0 109	8 21	2	1 711	4 144	1 10	9	92	41	5 143	0 84	8 17	5	1 6103	9 39	1 22	0	66	625	9		
VII	June 7	36	6 0155	0 108	6 21	6	1 210	4 141	8 13	2	96	47	5 140	5 84	5 17	9	0 9103	3 37	2 12	3	42	0 22	5		
VIII	" 28	30	7 3156	4 102	6 24	3	1 612	7 141	2 15	2	70	36	3 142	9 87	4 21	4	1 4110	2 32	7 7	5	62	8 26	5		
IX	July 7	31	7 2156	1 101	0 22	8	1 512	3 137	6 18	5	92	47	0 135	3 80	0 19	2	1 2100	4 34	9	8					
IX	Oct. 26	33	7 1155	5 106	2 21	6	1 412	3 141	5 14	0	114	49	0 136	0 78	2 16	8	1 3	96	3 39	7	9	4			
X	Nov. 1	24	6 7159	0 106	4 21	1	2 711	6 141	8 18	2	68	29	0 145	5 88	0 18	7	2 2108	9 41	7	9	9	47	639	854	8
XI	" 9	21	5 9158	8 106	8 22	9	1 910	2 141	8 17	0	91	37	0												
XII	" 16	31	6 0155	5 105	8 23	8	1 610	4 141	6 13	9	88	32	9						9	9					
XIII	" 22	27	6 7155	8 102	2 23	0	1 611	6 138	4 17	4	84	37	8						11	2	42	623	838	8	
XIV	" 29	28	5 8160	6 112	4 25	8	1 510	1 149	8 10	8	118	50	3						11	4					
Averages..		29	6 6157	9 105	9 23	0	1 811	3 142	1 15	8	91	40	6	142	0 84	3 19	1	1 6105	0 37	0	53	127	746	8	

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mange.

controlled diet. One notices at once the wide ranges of variation, especially in chloride, carbonate, protein, hematocrit, and hemoglobin. The differences far exceed the error limits of the methods and are in definite contrast to human blood which shows much less departure from averages. It is interesting to note that serum total base varies relatively less than the other electrolytes, eleven out of fourteen determinations between 155 and 159 m.-eq., with the limit of error ± 2 m.-eq. Total serum acid (sum of chloride, carbonate, phosphate, and protein ions), also, is surprisingly constant when one considers the uncertainties of the formulæ used in calculating the base-fixing powers; for example, eight out of fourteen observations are between 139.5 and 141.8 m.-eq. There is in the group, as a whole, no apparent consistent reciprocal or parallel relationship between the various electrolytes. The base in excess of that bound to determined acids (B-A) consists essentially of that bound to sulfate and organic acid (plus, perhaps, a certain amount of protein anion). This excess is surprisingly high. If one recalls the low concentration of sulfate (rarely more than 1 m.-eq.) the organic acid fraction appears so large that its nature is difficult to imagine.

Hematocrit and hemoglobin were roughly parallel. Two dogs (Nos. VIII and X) were anemic but in only one (Dog X) was the hematocrit strikingly reduced. Calculation of cell content from whole blood, serum, and hematocrit is subject to so much error that we have not included these figures. In general, whole blood and serum varied in similar fashion.

The results with Dog XIV (Table II) indicate that bleeding alone did not affect the protein per cent, hemoglobin, or hematocrit significantly. There was a parallel drop in base and acid of about 3.5 m.-eq., possibly unrelated to the blood loss.

The effect of complete fasting (fluids and food) was studied on two occasions (separated by 3 months) using the same dog. As can be seen in Table II there was no real concentration of the blood, according to the serum protein per cent, hematocrit, or hemoglobin; in fact, the protein per cent actually decreased in the first experiment. Chlorides varied much more than the other electrolytes, increasing (in serum) 12.4 m.-eq. and 5.0 m.-eq. Total base did not follow these changes (although it definitely increased), hence B-A decreased. When these results are compared with the

TABLE II
Control Dogs.

Dog No.	Date	Serum.										Whole blood.							Weight.	
		Non-protein N.	Protein	Total base	Cl	HCO ₃	PO ₄	Protein.	Total acid	B-A	Hb	Hematocrit	Total base	Cl	HCO ₃	PO ₄	Total acid.	B-A		
		mg per 100 cc	per cent	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	per cent	vol per cent	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	kg.	
IX ♀	1926 July 7 (Before)	31	7.2	156	1101	022.8	1.5	12	3137	618.5	92	47	0135	380	019.2	1	2100.4	34	9	8
	" 10 (After)	24	6.7	162	9113	420.8	2	011.7	147	915.0	92	47	0141	785	016.9	1	9103.8	37	9	3
IX ♀	Oct. 26 (Before)	33	7.1	155	5106	221.6	1	412	3141	514	0114	49	0136	078.2	16.8	1	3	96	339	7
	" 29 (After)	28	7.2	156	8112	221.5	1	912	5148	1	8	7116	49	8138	382	617.1	1	9101	636	7
XIV ♂	Nov. 29 (Before)	28	5.8	160	6112	425.8	1.5	10	1149	810	8118	50	3							
	Dec 2 (After.)	28	5.8	156	9110	624.5	1	310	1146	510	4116	47	1							
																			11.4	55 cc. blood removed. Regular food and fluid intake in interim.

findings in the dogs with ligated ureters, it seems apparent that the effects of bleeding and fasting play a relatively insignificant rôle.

TABLE III—Before and after

Dog No	Date	Serum												
		Non-protein N	Protein	Total base	Cl	HCO ₃	PO ₄	SO ₄	Protein	Total acid	B-A	Hb	Hematocrit	Total base
	1928	mg per 100 cc	per cent	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	per cent	vol per cent	m-eq per liter
II	Mar 22 (Before)	40	7.1	156.5	102.5	24.3	1.4		12.3	140.5	16.0		32.5	137.0
	" 25 (After)	219	7.0	152.0	84.4	17.4	10.3		12.2	124.3	27.7		32.5	132.0
III	Apr 12 (Before)	22	6.8	158.0	108.6	22.6	2.6		11.8	145.6	12.4		40.6	141.0
	" 16 (After)	282	6.8	158.0	77.3	28.5	8.1		11.8	125.7	32.3		39.5	150.0
IV	Apr 26 (Before)	29	6.6	161.3	105.0	22.2	1.8		11.4	140.4	20.9		43.0	141.8
	" 29 (After)	279	7.3	164.2	86.4	17.4	17.5		12.7	134.0	30.2		43.2	147.3
VI ♀	May 17 (Before)	27	6.6	155.0	109.8	21.2	1.7		11.4	144.1	10.9	92	41.5	143.0
	" 20 (After)	200	8.0	160.5	94.4	16.0	9.4	10.3	13.8	143.9	16.6	104	47.8	140.8
VIII ♀	June 28 (Before)	30	7.3	156.4	102.6	24.3	1.6		12.7	141.2	15.2	70	36.3	142.9
	July 1 (After)	213	6.9	148.0	83.2	20.0	6.4	15.6	12.0	137.2	10.8	64	31.7	138.0
X ♀	Nov 1 (Before)	24	6.7	159.0	106.4	21.1	2.7		11.6	141.8	18.2	68	29.0	145.5
	" 3 (After)	130	7.3	169.5	113.2	15.9	7.6		12.7	149.4	20.1	57	27.0	156.5
XIII ♂	Nov 22 (Before)	27	6.7	155.8	102.2	23.0	1.6		11.6	138.4	17.4	84	37.8	
	" 25 (After)	234	8.1	151.0	84.0	16.8	11.7		14.1	126.6	24.4	85	33.5	

The experimental animals (*i.e.* by operation) fall into three groups (Table III). (1) Dog X shows the early effects of renal insufficiency, (2) Dog III demonstrates the influence of excessive vomiting, and (3) Dogs II, IV, VI, VIII, and XIII developed fairly consistently the uncomplicated advanced uremia which we sought to produce. The early effect of ligation (Dog X) was a

marked increase in chloride and total base and moderate increase in phosphate, with almost equal decrease in carbonate. The levels of total base and total acid varied together so that there was

Ligation of Ureters.

Whole blood							Vomitus				Skin	Muscle	Brain	Weight	Fluid intake
Cl	HCO ₃	PO ₄	SO ₄	Total acid	B-A	Total amount	Total Cl	Total base	Cl-B	Cl	Cl	Cl	Cl		
m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	m-eq per liter	cc	m-eq	m-eq	m-eq	m-eq per kg	m-eq per kg	m-eq per kg	kg	cc	
86 0	21 2	1 7		108 9	28 1								12 7	<i>Ad libitum</i>	
72 6	14 7	8 8		96 1	35 9	189	25 0	17 0	8 0				9 1		
86 2	18 8	2 4		107 4	33 6									<i>Ad libitum</i>	
63 4	23 1	6 1		92 6	57 4	1945	218 0	140 6	77 4						
88 3	18 8	1 2		108 3	33 5								9 9	200	
69 8	15 3	11 7		96 8	50 5	480	18 6	15 9	2 7				8 8		
84 8	17 5	1 6		103 9	39 1					66 6	25 9		22 0	0	
70 0	13 0	6 5	10 9	100 4	40 4	165	31 9	31 8	0 1	67 6	8 9		20 2		
87 4	21 4	1 4		110 2	32 7					62 8	26 6		7 5	400	
71 6	17 6	4 9	16 0	110 1	27 9	220	34 5	25 1	9 4	52 5	12 2		6 8		
88 0	18 7	2 2		108 9	36 6					47 6	39 8	54 8	9 9	400	Considerable
94 0	14 6	6 2		114 8	41 7	110	4 1	3 8	0 3	60 2	35 2	54 2	8 9		bleeding at operation
										42 6	23 8	38 8	11 7	500	
						245	23 9	14 0	9 9	37 3	21 5	28 5	?		

little change in B-A. There seemed to be very little sulfate retention in the first stage. In other words, phosphate displaced carbonate while base and chloride increased together.

When excessive vomiting occurred (Dog III, Table III) we find the carbonate no longer yielding base to phosphate. The increase in phosphate and sulfate is compensated by the great loss of

chlorides in the vomitus. In spite of the vomiting, dehydration was negligible. Although analysis of total vomitus demonstrates that there is a real loss of base as well as chloride, yet surprisingly, there is no change in serum base to correspond with the great drop (31 m.-eq.) in chlorides.

Analysis of the cases in which an uncomplicated and advanced uremia was obtained (Dogs II, IV, VI, VIII, and XIII) is assisted by averaging the changes in the various electrolytes. The serum figures are:

Chloride	=	-17 9 m -eq	Phosphate	=	+9 4 m -eq
Carbonate	=	-5 5 "	Sulfate	=	+12 9 "
Total decrease	=	-23 4 "	Total increase	=	+22 3 "
Total base	=	-0 1 m -eq.	Protein	=	-1 1 m -eq

It is obvious that the significant alteration in the serum of these animals is the equimolar supplanting of chloride and carbonate with retained sulfate and phosphate. The most important factor in correcting the disturbance of the acid-base equilibrium is the mobility (in a physiological sense) of the chloride ion; this is true even when vomiting is negligible. It is rather interesting to note that the average increase in undetermined acid (B-A) is of the same order of magnitude as the average of the two sulfate determinations (average increase in sulfate was 12.9 m.-eq., average increase in B-A, 12.5 m.-eq.).

Total base moves moderately in both directions, averaging -0.1 m.-eq. There is a tendency for base to follow serum protein per cent (except Dog XIII), becoming more concentrated as water leaves the serum. This is contrary to present day ideas of its function in the fluid equilibria of the body, for it has been observed (10) that the body tends to alter fluid content in such a way as to keep total base constant. But there is sufficient inconsistency in the base figures to cast doubt on any definite conclusions. Altogether, the variations in total base are quite puzzling. There are so many unexplained swings beyond the apparent limits of error in the method, yet, as is pointed out above, in averages there is practically no change following ligation. It will be recalled that it was the most constant of all the normal figures.

The whole blood analyses add only the information that the

changes in the cells parallel those in the serum. There is no evidence that the red blood corpuscles receive any of the chloride that is displaced from the serum with the retention of sulfate and phosphate. The hematocrit and hemoglobin alter in the same direction but in three of the seven experimental animals hematocrit and serum protein moved in opposite directions. The result in Dog X may be due to hemorrhage at operation.

Study of the vomitus demonstrated that a large part of its chloride was bound to inorganic base. In only one instance was chloride minus base a significant figure. Dog III (Table III) lost 77 m.-eq. of Cl (next highest loss was 9.9 m.-eq.) and was the animal which showed such a marked decrease in serum chloride that carbonate was forced to replace it. In the other experiments Cl-B is so small that loss of chloride by vomiting can hardly be an important factor in explaining the rôle that chloride plays in the acid-base equilibrium. Dog VI for example, weighing 22 kilos, lost 15 m.-eq. of Cl per liter of whole blood although the base of the vomitus was practically equal to its chloride content. If one assumes the blood content in this animal to be 8.7 per cent of its weight, we have approximately 1900 cc. which, with a decrease of 15 m.-eq. per liter, would mean a total loss of 29 m.-eq. of Cl. But there is also an observed drop in muscle chloride to be added to this total loss. Even this total does not include a correction for the concentration of the blood indicated by the increased protein per cent (6.6 to 8.0 per cent), a correction which would add still more to the chloride loss.

Analysis of skin, muscle, and brain seems to indicate (Table III) that changes in chloride ions in these tissues tend to follow those in the blood. Omitting Dog X (where the blood chloride increased) there are seven analyses before and after operation, one on brain, three on muscle, and three on skin. Only one tissue (skin of Dog VI) did not decrease after ureteral ligation. The evidence is not in any sense conclusive, but, as far as it goes, it is evidence against storage of chloride in skin, muscle, or brain—unless there were a more than equal movement of water in the same direction. We hope in the future to include dry weights of the tissues.

The net effect on the blood serum of ligation of the ureters is, in résumé, a retention of phosphate and sulfate which takes base from carbonate and chloride. This equimolecular interchange

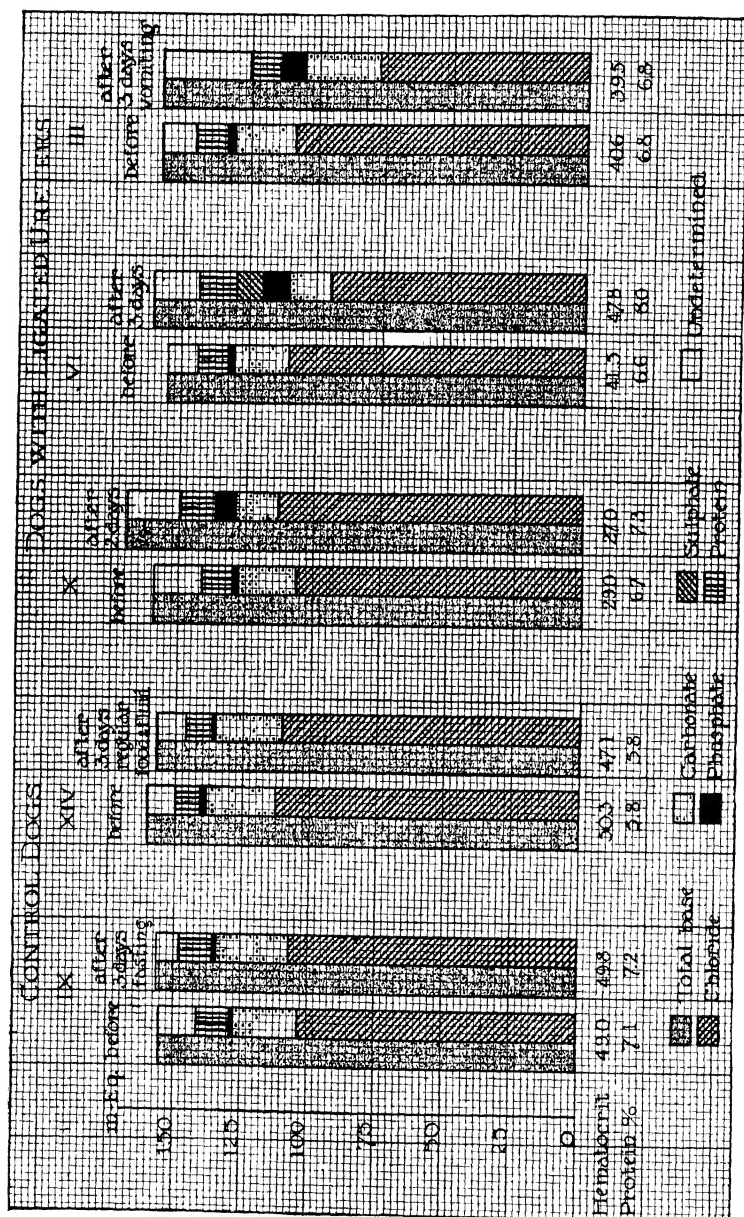


Fig 1.

occurs regardless of the movement of water to or from the tissues and seems to have no consistent effect on the total base content. The rôle that chloride plays in this mechanism is a very significant one; physiologically, it is much more mobile than carbonate in adjusting the acid increase imposed by renal insufficiency. In averages, it offers 3 times as much base as does carbonate. These changes are shown graphically in Fig 1

It is interesting to speculate on the mechanism of this phase of chloride metabolism. In normal animals, chloride may be eliminated from the blood stream without decrease in fixed base: (1) by the vomiting of HCl from the stomach (this occurred in Dog III), (2) by excretion of chloride in the urine in combination with ammonia formed by the kidneys, and (3) by change in the $\frac{\text{Cl serum}}{\text{Cl tissues}}$ ratio, due to changes in the Donnan equilibrium because of a movement of water to the tissues, thus altering the relative concentrations of non-diffusible electrolyte (protein). In our experimental animals we have controlled the first factor by analysis of vomitus, and found it to be insignificant in all but one case. The kidneys obviously do not function due to the ureteral ligation. It is impossible to eliminate the third possibility with the experimental evidence at hand. However, there are certain facts that seem to argue against it; first of all, there was no consistent change in the concentration of the serum and, in the second place, the possible effects of such a shift are probably too small to explain the phenomena we have described. Moreover, as we have seen, the tissues analyzed showed a drop in chloride instead of a rise. Even if water moved to the tissues, BCl must move with it so that there could hardly be actual dilution of tissue chloride. One other unexplored possibility may account for the chloride loss, namely, excretion into the kidney or its pelvis and concentration there because of the back pressure. This suggestion we intend to check by future analyses

Relating our results to clinical nephritis in human beings, one may infer that the acidosis of uremia is due to phosphate and sulfate retention. It also seems clear that the low blood chloride in this condition is not necessarily due to the vomiting that so frequently occurs.

SUMMARY.

A study has been made of the acute renal insufficiency produced in dogs by ligation of both ureters. Profound changes occur in the distribution of the electrolytes of the blood and tissues. The average increase in phosphates plus sulfates in the serum is 22.3 m.-eq., while the average decrease of chlorides plus carbonates, is 23.4 m.-eq., practically an equimolecular interchange. Total base of the serum shows several unexplained swings in both directions but the average change is negligible. Chloride yields to the retained acids 3 times as much base as does carbonate but in spite of its great importance in the acid-base equilibrium, no satisfactory explanation of its mechanism of action is apparent.

Whole blood changes parallel those of serum and most of the tissue analyses showed that tissue chloride changed in the same direction as serum chloride.

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THE MICRO DETERMINATION OF PENTOSE IN YEAST NUCLEIC ACID AND ITS DERIVATIVES.

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In recent years, pentoses, in combined form, have been found to be widely distributed in the animal organism. Inosinic acid has long been known to be present in muscle (1). Guanine nucleotide, adenine nucleotide, and cytosine nucleotide have been found in the β -nucleoprotein obtained from the pancreas (2). Adenine nucleotide, too, has been isolated from blood (3). This widespread distribution implies a definite rôle on the part of pentoses in the animal economy. A study of this rôle, however, has so far not been undertaken, chiefly because no attack is possible until a reliable, accurate, and convenient method is obtained of estimating minute amounts of pentose in free or combined form. The present investigation was undertaken to devise such a method, preparatory to a study of pentose metabolism. At the same time, a method having been devised, a study has been made of the pentose partition in yeast nucleic acid.

All modern methods of estimating pentose depend upon the conversion of pentose into furfural by distillation with 12 to 20 per cent hydrochloric acid. The furfural obtained has been determined in various ways, the most commonly used depending upon the precipitate formed by furfural with phloroglucinol in acid solution (4). Pervier and Gortner (5) in an excellent review of the literature, have shown the inaccuracy and infeasibility of all the methods proposed in the past for determining the furfural from pentose, and offer a new electrometric method, which gives, according to their figures, theoretical values for the estimation of pentoses. Their method, which depends on the oxidation of

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furfural by potassium bromate, the end-point determined electrometrically, aside from its inconvenience as a slow procedure and requiring electrometric apparatus, is not nearly so easily adaptable to micro technique as the colorimetric method recently offered by Youngburg and Pucher (6). The method of these investigators depends upon the formation of a red color when a furfural solution is mixed with aniline and acetic acid. The red color formation detects exceedingly small concentrations of furfural; and solutions which contain 0.00005 per cent or more of furfural can be quantitatively compared in a colorimeter. It is this method, with a number of modifications, which has been used here for the determination of pentose in free form or combined as nucleosides, nucleotides, or yeast nucleic acid.

Pervier and Gortner have shown that the chief difficulty in determining pentose has been that of obtaining a quantitative conversion of pentose into furfural by distillation with hydrochloric acid. The difficulty in the present investigation is even greater, for, besides the danger of destruction of furfural by long continued contact with hot hydrochloric acid, there is the danger of incomplete hydrolysis of the pentose-containing substances. Besides this, a more important consideration is the fact that, when dealing with quantities of furfural of the order of magnitude of 1 mg., small losses through oxidation by volatile substances in the steam such as chlorine, or by absorption by rubber stoppers, would make a considerable error in the determination. In line with the work of Pervier and Gortner, it was found that steam distillation of a reaction mixture containing hydrochloric acid of 12 to 20 per cent concentration would not only produce a complete hydrolysis of the pentose-containing substance with the formation of furfural, but also a quantitative transferal of the furfural formed,—if two other precautions were taken. These are first, that the water used as the source of steam contain a little potassium permanganate and sodium hydroxide to prevent the distillation of chlorine or other volatile substances; and secondly, that the furfural do not come in contact with rubber stoppers. The latter condition has been made conveniently possible by the use of a distilling flask with a mercury seal and with a long side arm serving as a condenser. Only under these circumstances could one obtain a quantitative distillation of a known weight of fur-

fural itself (say, 1 mg.) from a mixture containing 50 cc. of 20 per cent hydrochloric acid.

The technique of the colorimetric determination of the furfural in the distillate which is used by Youngburg and Pucher has been somewhat modified in order to give consistently accurate results. These authors take an aliquot portion of the distillate (1 to 5 cc.), neutralize to phenolphthalein with 50 per cent sodium hydroxide from a capillary pipette, add 0.5 cc. of aniline and 4 cc. of glacial acetic acid. The standard solution containing approximately the amount of furfural expected is treated similarly. The two solutions are then diluted to 10 cc., allowed to stand in a dark place for 15 minutes, and then compared in a colorimeter.

There are two difficulties with this procedure. In the first place, neutralization with 50 per cent sodium hydroxide is a very uncertain and annoying procedure. The end-point of two different solutions can scarcely ever be made the same, whereas the intensity of the color formed is quite dependent upon the pH of the solution. In the second place, unequal sodium chloride concentrations in the unknown and standard solutions produce colors which are not easily matched and which fade out at different rates. Thus it was found that two solutions, containing the same amount of furfural but one containing twice as much hydrochloric acid as the other, when neutralized to phenolphthalein with 50 per cent sodium hydroxide and diluted to 10 cc., gave readings in the colorimeter of 20 against 23.1. One faded in 20 minutes, the other in 35 minutes. Similar results were obtained in many other cases. At best, the colors were difficult to match.

Both of these difficulties can be avoided by the following modification of the technique. The distillate is collected in a volumetric flask holding a little more than the amount of liquid expected. The distillate is then titrated in this flask with 10 per cent sodium hydroxide from a burette to neutrality to phenolphthalein, and the liquid is then diluted to the mark. The standard solution equivalent to the amount of furfural distilled is placed in a flask of the same size and treated with hydrochloric acid and sodium hydroxide until the solution is neutral to phenolphthalein and the sodium hydroxide added equals the amount added to the unknown. This solution is also diluted to the mark. The two solutions now contain the same concentration of sodium chloride and are ex-

actly neutral to phenolphthalein. 6 cc. of each solution are transferred to a test-tube; each is treated with 0.5 cc. of aniline and 4.0 cc. of glacial acetic acid, allowed to stand for 10 to 15 minutes in the dark, and then compared in a colorimeter. This procedure gives quantitative results, the error in all the determinations varying from 0.5 to 3 per cent, which is that of most colorimetric determinations.

Determination of Xylose and Arabinose.—The method as outlined above was first tried on xylose and arabinose. The tables in the experimental part show that xylose gives quantitative yields of furfural when distilled from 50 cc. of 20 per cent HCl for 3 hours. Arabinose, on the other hand, is much more slowly converted into furfural. Under the conditions in which xylose gives theoretical yields, arabinose gives only a little more than 50 per cent yield. That the poor yield is not due to the method is indicated by the fact that 5 hours distillation of arabinose with 200 cc. of 20 per cent HCl gives practically theoretical results. This difference in the speed with which furfural is formed from xylose as compared with arabinose has been recognized by Pervier and Gortner.

The fact that, as will be seen, nucleotides furnish furfural at a much more rapid rate than arabinose suggested the idea that the presence of small amounts of phosphoric acid might catalyze the reaction, just as phosphoric acid catalyzes many other reactions of the sugars. Experiments, however, using varying amounts of phosphoric acid in the reaction mixture all failed to show any increase in the speed of furfural formation. The difference between xylose and arabinose is due to their molecular configuration.

Determination of Pentose in Nucleotides and Nucleosides—The difference between xylose and arabinose in the speed with which they are converted into furfural, indicates that in the study of *d*-ribose compounds, one has no way, from *a priori* considerations, of foretelling the speed with which they are converted into furfural, or whether they can under any circumstances furnish theoretical yields of furfural. The literature gives no reference to any quantitative determination of ribose-containing compounds by the formation of furfural. Herein lies the crux of the problem. For, any method of pentose estimation, to be of value in studies of pentose metabolism in the animal body, must prove to be efficacious, not only for xylose and arabinose, but especially for *d*-ribose, for it is as ribose

compounds that one finds pentose in the body. It was therefore, gratifying to find that chemically pure compounds of *d*-ribose which are readily hydrolyzed, such as the purine nucleotides and nucleosides, furnish the theoretical amounts of furfural when distilled with 20 cc. of 20 per cent HCl for 3 hours.

The fact that adenosine and guanosine, which are hydrolyzed to form the free pentose in a much shorter time than is adenine nucleotide or guanine nucleotide, still require 3 hours for quantitative production of furfural, shows that the time required is not to hydrolyze the nucleotide or nucleoside, but rather to convert the free ribose formed into furfural. The speed of this conversion is about the same as that of xylose.

The pyrimidine nucleotides are quite stable to the action of acids, and are hydrolyzed at an extremely slow rate of speed. It is therefore impossible to obtain theoretical yields of furfural from them. In a 3 hour distillation with 50 cc. of 20 per cent HCl, cytosine nucleotide gives 4.5 per cent of its theoretical amount of furfural, while uracil nucleotide gives 13.7 per cent. In studying uracil nucleotide the crystalline lead salt was used, as the only available pure derivative of uracil nucleotide. The presence of lead chloride might possibly have some influence in speeding up the conversion of pentose into furfural, but it is highly doubtful. It is much more likely that uracil nucleotide, though quite difficult to hydrolyze, is still more easily hydrolyzed than is cytosine nucleotide.

The small yields of furfural from pyrimidine nucleotides offer a convenient weapon for determining the purity of nucleotides. In the separation of the nucleotides from each other, guanine nucleotide is frequently found contaminated with cytosine nucleotide, whereas cytosine nucleotide is difficult to purify away from small traces of adenine nucleotide. The presence of a trace of a pyrimidine nucleotide with a purine nucleotide, or *vice versa*, is difficult to recognize by a nitrogen analysis, but can be easily discerned by determining the furfural obtainable by distilling for 3 hours with 50 cc. of 20 per cent HCl. For example, one sample of crystalline cytosine nucleotide, which had a nitrogen percentage of 13.70 instead of the theoretical 13.00 gave 12.8 per cent of the theoretical amount of furfural in 3 hours, of which two-thirds probably came from adenine nucleotide present as an impurity.

Study of the Pentose Partition in Yeast Nucleic Acid.—With a knowledge of the speed with which the individual nucleotides produce furfural, a study can be made of the pentose partition in yeast nucleic acid. Jones (7) some time ago studied the phosphorus partition in yeast nucleic acid, and was able to show that half the phosphorus is easily split off while the other half is only very slowly set free. Thus, he proved that the number of purine nucleotide elements in the molecule of yeast nucleic acid equals the number of pyrimidine nucleotide elements. In view of the recently expressed doubt (8), as to the primary existence of uracil nucleotide in yeast nucleic acid, it is desirable to obtain further evidence that the number of purine elements equals that of the pyrimidine elements. For, if this can be shown then proof of the absence of uracil nucleotide in yeast nucleic acid is conclusive only when more than 1 equivalent of cytosine nucleotide is found.

The present study shows very definitely that half the pentose is in combination with purine nucleotides and is easily split off, while the other half, in combination with pyrimidine nucleotides, is only very slowly set free. In 3 hours distillation with 50 cc of 20 per cent HCl, the ratio of molecules of furfural obtained to total phosphorus atoms for the purine nucleotides is 0.98, if one takes 98 per cent as the average of the yields obtained. For the pyrimidine nucleotides, using the average 4.5 per cent and 13.7 per cent yield of furfural, it is 0.091. The theoretical ratio, therefore, of furfural to total phosphorus for yeast nucleic acid for that time, assuming 2 purine molecules and 2 pyrimidine molecules, is 0.536. The actual ratio obtained for 3 hours is 0.513. The assumption made, therefore, that a molecule of yeast nucleic acid is made up of two purine groups and two pyrimidine groups is correct.

EXPERIMENTAL.

The apparatus used for the conversion of pentose into furfural is the ordinary steam distillation device, except that the distilling flask has no rubber stoppers. Instead, the side arm of the flask was made quite long so as to serve as the inner tube of a condenser, and had attached over it the jacket of a condenser. The tube that runs from the steam generator into the distilling flask is sealed by

mercury with the flask. Several sizes of apparatus were tried out with the purpose of obtaining a minimum amount of distillate for a 3 hour run. A 500 cc. distilling flask was found most convenient to hold the reaction mixture. The speed of the steam production was minimized by using a small flask as a generator. Distilled water containing potassium permanganate and sodium hydroxide was used as a source of steam.

TABLE I
Xylose and Arabinose

Quantity determined	20 per cent HCl in reaction mixture	Time of distillation	Xylose recovered as furfural
Xylose			
<i>mg</i>	<i>cc</i>	<i>hrs</i>	<i>per cent</i>
0 5	20	1 $\frac{3}{4}$	86 3
0 5	15	2	91 9
1	15	3	97 3
10	50	2	95 0
10	50	2	91 9
10	50	3	98 8
10	50	3	98 8
10	50	3	96 0
10	50	3	100 8
10	50	4	99 8
Arabinose			
10	50	3	52 8
10	50	3	54 4
10	100	5	82 3
10	150	4	87 4
10	150	5	90 9
10	200	5	98 8

Steam distillation of furfural itself is complete within a half hour, so that the 3 hours required for complete production of furfural from xylose and from ribose compounds is necessary in order to convert the pentose into furfural. Pervier and Gortner have shown that the amount of distillate is unimportant, a slow stream of steam being just as effective as a fast one. The temperature of the reaction mixture however is important. It should be kept, as Pervier and Gortner say, at 103–105°, for, at higher temperatures,

TABLE II.
Nucleotides and Nucleosides.

Quantity determined	20 per cent HCl in reaction mixture	Time of distillation	Theoretical furfural obtained
Adenine nucleotide.			
<i>mg</i>	<i>cc</i>	<i>hrs</i>	<i>per cent</i>
10	50	2	92 3
10	50	2	92 7
5	50	2	86 6
5	50	2	88 2
5	50	3	95 0
10	50	3	98 7
10	50	3	98 9
10	50	4	97 2
Guanine nucleotide			
4 41	50	1	69 7
8 82	50	1	76 4
8 82	50	1½	82 0
8 82	50	2	89 2
8 82	50	2	85 6
8 82	50	2	91 5
8 82	50	3	98 4
8 82	50	3	97 4
Adenosine			
5	50	1	65 0
10	50	2	88 6
10	50	3	99 8
10	50	3	97 8
Guanosine			
10	50	1	77 8
5	50	1½	91 2
10	50	1½	90 7
10	50	2	95 0
10	50	3	97 4
10	50	3	97 4
Cytosine nucleotide			
15	50	1	1 62
10	50	1½	2 19
10	50	1½	2 57
10	50	2	3 06
20	50	2½	4 08
17	50	3	4 47
20	50	3½	5 07
20	50	4	5 98

TABLE II—*Concluded*

Quantity determined.	20 per cent HCl in reaction mixture	Time of distillation.	Theoretical furfural obtained
Uracil nucleotide *			
<i>mg</i>	<i>cc</i>	<i>hrs</i>	<i>per cent</i>
50	50	1	6 35
50	50	1	5 98
50	50	2	10 25
50	50	2½	11 20
50	50	3	14 00
50	50	3	13 40
50	50	4	16 65

Ratio of $\frac{\text{Mols furfural obtained in 3 hrs}}{\text{Mols total phosphorus}} = 0.0447$ for cytosine nucleotide.
 " " " " = 0.1370 for uracil nucleotide.

Mean ratio = 0.091.

* Crystalline lead uracil nucleotide was used

TABLE III

Yeast Nucleic Acid

Each mg of yeast nucleic acid contained 0.081 mg of phosphorus.

Amount of yeast nucleic acid determined	Time of distillation (50 cc of 20 per cent HCl used in each case)	Furfural obtained	$\frac{\text{Mols of furfural}}{\text{Mols of total phosphorus}}$
<i>mg.</i>	<i>hrs</i>	<i>mg.</i>	
40	1	4.39	0.403
40	1	4.53	0.416
40	2	4.84	0.445
40	2	4.99	0.458
40	3	5.61	0.515
40	3	5.55	0.510
40	3	5.61	0.515
40	3	5.55	0.510
40	3	5.58	0.513
40	4	5.69	0.523
40	5	5.76	0.529

Average for 3 hrs. of $\frac{\text{mols of furfural}}{\text{mols of total phosphorus}} = 0.513$

Theoretical ratio = $\frac{0.98 + 0.091}{2} = 0.536$.

decomposition may occur and besides the hydrochloric acid is soon distilled out, and at lower temperatures the volume in the distilling flask increases, producing a diminution of the concentration of the hydrochloric acid and a consequently decreased speed of reaction. This constancy of temperature can be acquired with a little practice without the use of a thermometer by regulating the size of the flame under the distilling flask. Good results are obtained if the volume is allowed to remain constant or to decrease very slowly. In 3 hours, under such conditions, the concentration of hydrochloric acid never gets below 12 per cent.

The reagents used for the colorimetric determination should be, as Youngburg and Pucher show, of the highest purity. The aniline was always freshly distilled, and the furfural used for standard solutions was repeatedly distilled under reduced pressure. The standard solutions were always made up with toluene-saturated water to prevent bacterial decomposition.

Xylose and arabinose were Pfanstiehl products of the highest purity. They had specific rotations respectively $+18.5^\circ$ and $+104.5^\circ$. The nucleotides and nucleosides used were all pure products obtained by hydrolysis of yeast nucleic acid. They were furnished the author by Dr. M. V. Buell and Dr. H. O. Calvery of this Department, to whom the author wishes to express his thanks. The guanine nucleotide used was in crystalline form, obtained by the method of Buell and Perkins (9). The yeast nucleic acid was a Behrenger product.

Tables I to III give a summary of some of the results obtained. They show that the method gives theoretical results in 3 hour distillations using 50 cc. of 20 per cent HCl in the case of xylose, adenosine, guanosine, adenine nucleotide, and guanine nucleotide. Arabinose is only slowly converted into furfural, and the pyrimidine nucleotides are only very slowly hydrolyzed. Yeast nucleic acid gives up a little more than half of its pentose in 3 hours, as is to be expected.

SUMMARY.

A micro method of determining pentose in free or combined form has been devised, which is based on the methods of Pervier and Gortner and of Youngburg and Pucher.

The method has been found to give theoretical results with com-

pounds containing *d*-ribose, and is therefore applicable to a study of the pentoses in the body.

A study of the pentose distribution in yeast nucleic acid confirms the prevailing idea that yeast nucleic acid contains the same number of purine elements as it does pyrimidine elements.

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N METABOLISM IN THE PUERPERIUM.*

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It is usual to regard the puerperal period as marked by a N loss. The early experiments of Grammatikati (1), Zacherjewski (2), etc., and the more recent ones of Slemons (3), Murlin (4), and Wilson (5), have made it evident that a diet which, under ordinary circumstances, would be adequate in calories and protein, and which in fact produces large N gains in the latter half of pregnancy, proves itself inadequate to maintain a N balance in the puerperium. It is usual to ascribe this N loss to the involution of the uterus. The atrophic changes which this organ undergoes, changing from roughly 1000 gm. to 50 to 100 gm. in the space of a fortnight are striking, and it cannot be doubted that amino acids set free by autolysis in this process will find their way into the urine as urea during a period of N loss. Zacherjewski appears to have been the first to particularize the involution of the uterus as responsible for the N loss, but it was emphasized vigorously by Slemons, who compared the N loss in a case of simple cesarean section with that in which the uterus was removed. The case of simple cesarean section lost 2 to 3 gm. more N than the other, an amount comparable with that expected, were the extra loss due to the involution of the uterus. Grammatikati alone appears to have offered another explanation of the N loss, ascribing it to the process of lactation. Under the conditions of his experiments he

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observed a maximum output of urea coincident with the appearance in the breast of a heavy milk flow, and a low urea output in those cases nursing irregularly. He explained his results as due to the conversion of protein to the fat of the milk with the liberation of the N into the urine. In our opinion the inadequacy of his explanation has obscured the accuracy of his observations.

The origin of the N loss is also of interest in its bearing on the problem of postpartum creatinuria. Shaffer (6) first observed this phenomenon in women, and Murlin (7) in dogs. Here again the involuting uterus would appear to offer a simple solution of the origin of the urinary creatine at this time. The amount of creatine excreted however is often in excess of that expected were the creatine to arise solely from the autolyzing uterus. Thus Shaffer observed as high as 1.5 gm. of creatine a day. Beker's (8) calculations, based on a creatine content of uterine muscle of 89 mg per 100 gm., make only 0.73 gm. of total creatine. This amount is probably too low judging by the experience of Harding and Eagles (9) who found the creatine content of brain to be 100 to 116 mg per 100 gm. compared with the figure of 51 to 63 mg. per 100 gm. of Beker, but even making every allowance for error, 1.5 gm. represents a maximum amount of uterine creatine. Mellanby (10), assuming the uterus, as a plain muscle, contained little or no creatine, advanced the view that lactation should be regarded as responsible for the creatinuria. He observed a parallel between the creatine-creatinine ratio and the rate of growth of the breast-fed babies; delayed lactation corresponded to a delayed creatinuria; early lactation and early creatinuria were also associated. One must observe here, however, that creatinuria is often found in late pregnancy. Morse's (11) experiments on the creatinuria of simple cesarean section and cesarean section accompanied by hysterectomy, convinced him that the origin of postpartum creatinuria was not in the uterus, though he refused to accept Mellanby's explanation. The histories of the N balance and of the creatinuria of the puerperium thus offer an interesting parallel.

Is the N loss of the puerperal period inevitable? Previous experiments have simply observed the change of N balance on diets, which should have been adequate under ordinary conditions. Can diets, containing a sufficient amount of N and an excess of calories, cause N gains? Gains such as these could, of

course, be attained, either by checking the involution of the uterus, or masking the N losses consequent on its involution by N gains in other parts of the body. Experiments involving a similar problem have been common in the field of N losses in febrile conditions. The feeding of high calory diets at a sufficiently high level of N intake has resulted in N gains in typhoid fever (12) and tuberculosis (13) without deciding how such gains are actually attained. Experiments on N balance during the involution of the uterus have the advantage that the organ can be measured by a pelvimeter and thus a rough idea of the atrophy obtained by independent means. The results also are not complicated by fluctuating temperature. It is true that the involution of the uterus represents a localized occurrence, and that its contraction after labor is sharp and rapid, thus cutting off some of its blood supply before dietary influences have time to come into play; nevertheless the acute atrophic processes continue some 14 days, giving ample opportunity for observation. The course of the N balance under these conditions might also be expected to supply further evidence on the creatinuria.

With such ideas in mind we have carried out experiments on four selected cases, placing each subject on a diet ample in calories and N intake immediately after labor. All subjects were normal, had been under observation some 3 or 4 months previous, passed through a normal labor and returned from the delivery room in excellent condition. Immediately, 2000 cc. of 10 per cent glucose in normal saline were given intravenously and food was urged in the first 24 hours. There was thus no period of starvation. The subjects remained in bed during the whole of the observation period. Three diets were used, 2500, 3000, and 4000 calories respectively and containing 25, 23, and 34 gm. of protein. The 4000 calory diet was used for two subjects (D-k-n and W-r). The subjects received 38, 52, 54, and 74 calories, and 0.079, 0.092, 0.094, 0.14 gm. of N per kilo of body weight respectively. The amount of N is rather low according to ordinary standards except in the last case, but should have been sufficient to cover the ordinary wear and tear N in view of the calories supplied; 6 gm. of salt were given daily. The food was constant throughout the experiment. The details of the diets are to be found in Table I.

Urine was collected daily, preserved under toluene, and analyzed

TABLE I.

Food	Diets.		
	I	II	III
	gm.	gm	gm
Breakfast.			
Stewed figs	80	130	150
Rolled oats (dry weight)	20	30	30
Marmalade	30	30	40
Bread	40	40	50
Butter	20	20	25
Cream, 32 per cent	60	60	80
Sugar	30	30	30
Dinner			
Grape juice with 10 gm sugar			210
Chicken			40
Potato	100	120	130
Vegetable (I, 10 per cent) (II, 15 per cent)	100	100	
“ salad (5 per cent) ..	75	100	
Corn			120
Lettuce with 50 gm banana (edible portion)			90
Bread	30	40	50
Butter	12	15	25
Cream, 32 per cent	80	80	100
Sugar	30	30	30
Rice	100	120	100
Raisins	30	40	40
2 30 p m. 100 gm orange juice with 10 gm. sugar			110
Supper			
Baked potato	200	200	200
Vegetable, 10 per cent	100	100	
“ 5 “ “ ..	75		
Lettuce		40	40
Orange		50	60
Parsnips			100
Bread	30	40	50
Butter	13	10	18
Cream, 32 per cent	60	60	100
Honey	30	45	
Sugar	28	30	19
Baked apple			150
8 p m. Grape juice		200	200
Sugar			10
Carbohydrate	375	501	628
Fat	99	100	150
Protein	25	23	34
Calories	2497	3000	4001

TABLE II
N Balance

Case M-1-r Gravida I, age 19 yrs, height 5 ft 4½ in, weight 144½ lbs Labor, Feb 21, 1926

Date	Weight of baby	Urine		Lochia N	Feces N	Milk		Total N	Food N	Balance* N	Blood analysis				Polymer readings.	
		Vol- ume	cc	N	gm	cc	gm	gm	gm	gm	Date	Non- protein N	Urea N per 100 cc	Serum pro- tein	Date	cm.
Feb. 22	3742 0	3700	17 09	0 22	2 23			18 94	-13 77		Feb. 23	25 1	11 65	5 25	Feb 22	9
" 23	3628 8	2150	9 93	0 21	0 69	134 9	0 34	12 11	-6 94		"	25	20 2	5 25	" 23	11
" 24	3642 9	2420	10 09	0 36	2 70	227 2	0 58	12 66	-7 50		"	27	20 5	5 70	" 24	10
" 25	3664 1	1260	5 36	0 16	1 88	340 8	0 92	8 07	-2 90		Mar	1	22 4	5 68	" 25	10
" 26	3727 7	1550	8 85	0 15	0 98	397 6	0 81	11 44	-6 27		"	3	21 6	6 12	" 26	10
" 27	3756 0	1930	6 05	0 31	2 57	397 6	0 81	8 80	-3 63		"	5	20 2	5 47	" 27	9
" 28	1520	4 70	0 37	0 32	0 27	333 7	0 72	7 42	-2 26		"	8	20 4	5 12	" 28	8
Mar. 1	3713 7	1200	5 30	0 30	3 08	269 8	0 69	8 56	-2 78		"	10	20 8	6 55	Mar 1	5
" 2	1400	5 94	0 30			333 7	0 56		-3 40						" 2	S. P.
" 3†	3727 7 (150)															
" 4	3798 6	1450	5 35	1 27	1 39	298 2	0 60	7 58	-2 41							
" 5	3826 9	1350	4 76	1 39	1 57	291 1	0 61	7 00	-1 83							
" 6	3826 9	760	4 37	1 57	1 70	440 2	0 91	6 91	-1 74							
" 7		870	3 80	1 70	0 64	426 0	0 94	6 37	-1 20							
" 8	3826 9	1070	2 74	0 64	1 74	380 5	0 84	5 21	-0 04							
" 9	3826 9	1500	3 46	1 74		383 4	0 80	5 89	-0 72							
Totals			97 79	2 40	22 70	4998 4	10 84	134 92	82 72	-57 39						

* In calculating the N balance the average figures of 1 63 gm of fecal N and 5.17 gm of food N are used.

† Temperature 102-103°, congestion of mammary gland No urine collections.

S. P. = At level of symphysis pubis.

TABLE III.

N Balance.

Case B-1-n Gravida 2, age 20 yrs, height 5 ft 3½ in, weight 127 lbs Labor, Mar 20, 1926

Date.	Weight of baby	Urine		Lochia N	Feces N	Milk		Total N	Food N	Balance* N	Blood analysis				Pelvimeter readings	
		Vol-ume	N			Vol-ume	N				Date	Non-protein N	Urea N per 100 cc	Serum pro-tein	Date	
	gm	cc	gm	gm	gm	cc	gm	gm	gm	gm		mg	mg	per cent		cm
Mar 23	2664 2	1360	7 17	0 41	1 42	140	0 41	9 53	5 46	- 4 30	Mar 23	18 8	8 15	5 68	Mar 22	8
" 24	2678 3	1450	5 68	0 43	1 03	318	0 93	8 58		- 3 34	" 25	19 6	7 68	6 12	" 23	8
" 25	2664 2	1050	5 93	0 71	1 04	318	0 93	9 11		- 3 87	" 27	20 8	9 46	6 12	" 24	7
" 26	2710 6	1200	4 70	0 53	1 29	279	0 88	7 65		- 2 41	" 29	19 7	9 32	6 55	" 25	6
" 27	2806 5	1400	6 17	0 52	2 71	407	1 27	9 50		- 4 26	" 31	21 2	8 12	8 49	" 26	5
" 28		950	5 15	0 47	2 03	294	0 91	8 07		- 2 83	Apr 2	19 8	7 45	8 25	" 27	5
" 29	2841 8	870	4 81	0 00	1 46	365	1 15	7 50		- 2 26	" 6	19 1	6 83	7 89	" 28	4
" 30	2884 2	950	5 22	0 27	1 88	211	0 66	7 69		- 2 45	" 9	29 8	16 40	7 42	" 29	3
" 31	2933 8	1020	4 41	0 24	1 75	349	1 09	7 28	5 03	- 2 04	" 12	30 6	20 50	8 49	" 30	3
Apr. 1	2962 1	1040	4 16	0 13	1 05	451	1 42	7 24		- 2 01	" 14	26 9	18 17	7 73	" 31	S P.
" 2		1420	3 67	0 16	1 80	414	1 30	6 66		- 1 43						
" 3	3132 9	(700)	3 22	0 09	1 03	367	1 16	6 01		- 0 77						
" 4		1250	3 29		0 88	368	1 16	6 00		- 0 75						
" 5	3089 5	1060	3 47		1 03	433	1 33	6 34	5 25	- 1 10						
" 6	3153 1	950	3 16		2 78	509	1 60	6 30		- 1 06						
Totals			70 21	3 96	23 18	5223	16 20	113 48	78 60	- 34 88						

* In calculating the N balance the average figures of 1 54 gm of fecal N and 5 24 gm of food N are used.

TABLE IV.

N Balance.

Case D-k-n. Gravida 2, age 23 yrs, height 5 ft 4½ in, weight 165½ lbs. Labor, Apr 24, 1926.

Date.	Weight of baby	Urine		Lochia N.	Feces N	Milk		Total N	Food N	Balance* N	Blood analysis.				Pelmeter readings			
		Vol-ume	N			cc	gm				gm	gm	Date	Non-protein N	Urea N per 100 cc	Serum pro-tein.	Date	cm
Apr 25	Still born	1340	6 96	0 40	2 24			9 61	6 38	-2 61	Apr 27	12 86	5 86	6 34	Apr 24	11		
" 26		1250	4 02	0 17	1 35			6 44	7 20	+0 56	" 29	14 80	5 98	6 44	" 25	9		
" 27		1200	4 27	0 16	2 32			6 68	7 53	+0 32	May 1	18 60	6 76	6 98	" 26	10		
" 28		1040	5 02	0 42	2 21			7 69		-0 69	" 4	17 90	7 78	6 98	" 27	9		
" 29		1750	4 46	0 20	2 45			6 91		+0 09	" 6	10 60	4 66	7 20	" 28	8		
" 30		1420	3 67	0 28	1 76			6 20		+0 80	" 7	16 80	4 66	7 42	" 29	8		
May 1		1420	4 19	0 24	2 24			6 68		+0 32	" 10	16 20	6 99	6 98	" 30	8		
" 2		1000	3 74	0 23	2 93			6 22		+0 78					May 1	7		
" 3		1420	3 96	0 16	2 28			6 37	6 86	+0 63					" 2	5		
" 4		1190	3 57	0 04	2 17			5 86	6 94	+1 14					" 3	S. P		
" 5		1480	4 45	0 03	2 21			6 73		+0 27					" 4	"		
" 6		1900	4 17	0 12	1 97			6 54	7 11	+0 46								
" 7		1950	4 17		2 06			6 42	7 36	+0 58								
" 8		1500	3 82	0 14	2 38			6 21		+0 79								
" 9		1550	3 25		3 24			5 50		+1 50								
" 10		1580	3 51		1 88			5 76		+1 24								
" 11		1730	3 65		2 61			5 90		+1 10								
Totals.			70 88	2 59	38 30			111 72	119 0	+10 58								

* In calculating the N balance the average figures of 2 25 gm. of fecal N and 7 0 gm. of food N are used.

TABLE V.

N Balance

Case W-r Gravida 1, age 19 yrs, height 5 ft 2½ in, weight 118 lbs Labor, May 22, 1926

Date	Weight of baby gm	Urine		Lochia N	Feces N	Milk		Total N	Food N	Balance* N	Blood analysis			Polymer readings	
		Vol- ume cc	N			Vol- ume cc	N				Date	Non- protein N	Urea N per 100 cc	Serum pro- tein per cent	Date
May 23		1850	8 44	0 76				11 00	7 44	-3 32	May 24		11 2	5 47	May 23
" 24		1920	8 04	0 42	0 21		0 25	10 52		-2 83	" 27		10 3	7 25	" 24
" 25	3075 3	1180	5 81	0 57		670	1 62	9 81		-2 12	" 31		10 3	6 34	" 25
" 26	3131 9	1000	5 74	0 83	0 63	925	1 76	10 14		-2 45					" 26
" 27	3153 1	1000	6 49	0 95	1 37	975	1 55	10 80		-3 10					" 27
" 28	3153 1	1050	7 73	0 56		835	1 67	11 77	7 86	-4 08					" 28
" 29	3097 8	1031	5 91	0 25	3 94	815	1 56	9 53		-1 84					" 29
" 30		1000	4 57	0 13	3 73	690	1 29	7 80		-0 11					" 30
" 31	3198 3	1000	4 53	0 19	1 85	905	1 59	8 12		-0 44					" 31
June 1	3271 1	1000	4 91	0 19	0 99	865	1 52	8 43		-0 74					
" 2	3271 1	1000	4 90	0 15	2 37	712	1 18	8 04	7 78	-0 35					
" 3	3401 6	1000	5 01	0 13	1 97	615	1 08	8 03		-0 34					
" 4	3462 6	1000	4 32	0 08		575	1 11	7 32		+0 37					
" 5	3528 9	1000	4 59	0 16	1 59	715	1 34	7 90		-0 20					
" 6		1000	6 02	0 14	2 10	530	0 96	8 94		-0 20					
" 7	3543 1	1000	5 19		1 30	670	1 23	8 23		-1 24					
" 8	3599 7	1000	5 20			510	0 94	7 95		-0 54					
" 9	3642 8	1000	4 55			690	1 32	7 68		-0 26					
Totals.			101 95	5 51	22 05	11,822 21	97	162 01	137 42	-24 15					

* In calculating the N balance the average figures of 1 81 gm of fecal N and 7 69 gm of food N are used.

for N by the Kjeldahl-Gunning method. Lochia was collected on N-free pads; the pads collected daily and allowed to hydrolyze in warm 20 per cent sulfuric acid. The hydrolysate was made up to a definite volume and an aliquot portion used for N determination. Feces were obtained by a daily enema, placed in a jar, emulsified with warm 20 per cent sulfuric acid made up to 2000 cc., and 50 cc., were used for N determinations. The food N was determined by obtaining on three separate occasions, for each subject, a series of duplicate trays of the day's supply from the diet kitchen. The contents were well mixed, heated from 12 to 24 hours on a water bath with 20 per cent sulfuric acid until thoroughly disintegrated and emulsified, made up to 2000 cc., and 25 cc. sample used for N determination. A specially calibrated wide tipped pipette was used in obtaining this sample. Milk N was determined on 5 cc. by the Kjeldahl-Gunning method. The details of the N balance of each case are to be found in Tables II to V. Creatine and creatinine were determined by the method of Folin (14). Routine blood analyses were made from time to time throughout the experiment as shown in the Tables.

RESULTS AND DISCUSSION.

In no case were we able to affect measurably the rate of involution of the uterus. Even in the case of W-r, with an intake of 74 calories per kilo of body weight and 7.69 gm. of N, the involution of the uterus was complete in the usual time.

In only one case (D-k-n, Table IV) were we able to observe N gains. Marked losses of N occurred in the other three cases. The N gains of D-k-n occurred with an intake of 54 calories per kilo of body weight and 7.6 gm. of N. Normal involution measurements showed the autolytic processes in the uterus evidently continued at their usual rate, and one may calculate from an approximate weight of the uterus (1000 gm.) that at least 40 gm. of N in the form of tissue were broken down. Yet this was entirely masked by the influence of the diet which brought about a total gain of 2.45 gm. of N in 17 days. With the exception of 3 days, N gains were made steadily throughout the period of the active involution of the uterus, and as two of these losses were immediately following parturition it is reasonable to suppose that the diet had not had time to exert its maximum protein-sparing

effect. Some objection perhaps may be raised that the N of the uterus was retained in some form other than synthetic. If so, this form at least was not urea as judged by the concentration of that substance in the blood. The serum proteins rose but this is usual during a normal puerperium and similar rises can be found in the other cases.

The other three cases all show continuous N losses. The case of M-l-r (Table II) may perhaps be discarded as in comparison with D-k-n she was on a lower plane of intake both of calories and N, though both calories (38 per kilo) and N (5.17 gm) would be judged amply sufficient under ordinary conditions. We do not believe this to be the explanation of the N loss, but no such criticism can be advanced against cases B-l-n and W-r (Tables III and V). In the former the calory value and N content of the diet were the same per kilo of body weight as for D-k-n. In the latter both values were markedly higher. The conditions were such that the N set free from the involuting uterus should either have been utilized or its loss compensated by a new N gain. The difference in result can be attributed only, we think, to lactation. In D-k-n the baby was born dead through an accident, and lactation was suppressed by bandaging. The comparison forces us to conclude that the initiation of the process of lactation is attended by a N loss. Part of the loss is due to the actual milk N itself, but even if this is allowed for, as shown in Table VI, negative balances are still obtained. M-l-r and B-l-n do not show positive balances until the 15th and 14th day after delivery respectively. W-r, on the very high calory diet, with increased N intake, shows a positive balance on the 8th day if we exclude the milk N. It is possible that under the conditions of lactation a positive N balance may never be attained, for increases in protein intake or increases in calories will only result in an increased flow of milk, if one may judge by the experimental evidence on dairy cows. Meigs (15) in his excellent review of this subject has cited the literature leading to such a conclusion, though we find the N content of the milk of W-r to be 0.185 per cent in contrast with M-l-r and B-l-n with a milk N percentage of 0.217 and 0.310 respectively (*cf.* volumes of milk in Tables II, III, V). On the other hand, it would appear that a positive balance might be attained with a higher intake of N throughout the puerperium were we to

exclude the milk N from our calculations as an excretory product. Such a postulate is not usual, yet the formation of milk proteins represents a series of reactions differing totally from those leading to the formation of such nitrogenous excretory products as urea and properly classed as catabolites. The calculation of the N balance of the mother under such conditions would, however, be justified if we took into account the metabolic relation to the baby. Milk merely represents an intermediate in the nutritional

TABLE VI.
Daily N Balances Excluding Milk.

Days after labor.	M-l-r N	B-l-n N	W-r N
	gm	gm	gm
1	-13 77		-3 32
2	-6 60		-1 78
3	-7 92	-3 89	-0 50
4	-1 98	-2 41	-0 69
5	-5 46	-2 94	-1 55
6	-2 82	-1 53	-2 41
7	-1 54	-3 99	-0 28
8	-2 07	-1 92	+1 18
9	-2 71	-1 11	+1 15
10		-1 79	+0 78
11	-1 81	-0 95	+0 83
12	-1 22	-0 50	+0 74
13	-0 83	-0 13	+0 74
14	-0 26	+0 39	+1 14
15	+0 80	+0 41	+0 76
16	+0 08	+0 23	-0 01
17		+0 54	+0 40
18			+1 06

relation of the mother and child, and a determination of the N balance of the puerperium should include a study of this aspect. Otherwise, we must conclude that the process of lactation represents a synthesis on the part of the group of cells of the maternal organism, attended usually by a N loss even under conditions where the N of the uterus would appear to be available.

Later in the lactation the maternal organism goes into N equilibrium on an adequate diet, and one may well question the mechanism which forces a N loss on the body as a whole when one group

of cells commences a synthetic process. The N of the uterus may take part in the synthesis of milk proteins. The N stored in the latter half of pregnancy in excess of the needs of the fetus, hypertrophy of the uterus, mammæ, etc., may also be utilized for synthesis at the commencement of lactation. Hoffstrom (16) first suggested in a general way that this N might be utilized for milk

TABLE VII
Postpartum Creatinuria

Days after labor	D-k-n			W-r		
	Creatine + creatinine as creatinine	Creatinine	Creatine as creatinine	Creatine + creatinine as creatinine	Creatine	Creatine as creatinine
	gm	gm	gm	gm	gm	gm
1	1 34	1 09	0 25	1 72	1 32	0 40
2	0 72	0 59	0 13	1 87	1 12	0 74
3	1 12	0 98	0 14	1 49	1 04	0 45
4	1 16	1 05	0 11	1 06	0 86	0 21
5	1 31	1 10	0 22	1 14	0 77	0 36
6	1 39	1 14	0 25	1 73	1 25	0 48
7	0 98	0 98	-0 01	1 63	1 00	0 63
8	1 20	1 04	0 16	1 51	0 90	0 61
9	1 03	1 00	0 03	1 63	0 97	0 66
10	1 15	1 18	-0 03	1 50	0 92	0 58
11	1 18	1 11	0 06	1 50	1 07	0 41
12	1 13	1 06	0 08	1 47	1 06	0 41
13	1 23	1 10	0 13	1 44	1 00	0 45
14	1 37	1 20	0 20	1 31	0 99	0 33
15	1 11	1 02	0 08	1 35	1 10	0 28
16	1 15	1 11	0 05	1 48	1 20	0 30
17	1 25	1 13	0 12	1 26	1 10	0 16
18				1 04	1 06	-0 01
Totals			2 01			7 46

production, but its more particular use may be to act as a reserve store of N, anticipating the N loss inevitable under ordinary conditions of diet at the commencement of lactation. Our experiments justify to some extent the conclusion drawn by Grammatikati on a relationship between the N losses of the puerperium and lactation.

If the origin of the N loss is to be ascribed to the process of lactation rather than to the involution of the uterus, a similar reason would account for the phenomenon of postpartum creatinuria, and Mellanby's general conclusion would be upheld. Our measurements of the creatinuria were confined to two cases—D-k-n and W-r. The results are shown in Table VII. Both cases showed creatinuria, D-k-n on a positive N balance excreted slightly over 2 gm. during the 17 days of observation. This amounts to an average of 0.118 gm. per day. The excretion, however, is irregular, especially after the 6th day, minus quantities being found on the 7th and 10th days, and one cannot help but suspect that some of the analytical figures do not represent creatine. Such being the case the amount of creatine excreted puerperally might be no more than could reasonably be expected to arise from the involuting uterus.¹ In any event one must conclude that creatine set free by the breakdown of tissue is not immediately utilizable, though the subject may be in positive N balance. The case of W-r presents a complete contrast: the total creatine excretion amounts to nearly 7.5 gm. The excretion is sustained throughout the observation period until the 18th day. The amounts are so large that one can hardly doubt the presence of a sustained creatinuria. Here the difference between the lactating and non-lactating puerperium makes itself evident again. What is the origin of this extreme outpouring of creatine? It cannot all have arisen from the uterus. Has it arisen from the breakdown of the nitrogenous tissue occurring at the commencement of lactation? If so, such a breakdown must have continued for 16 to 17 days though the N balance (excluding milk N) indicated storage after the 8th day. Such an interpretation involves the masking of a N breakdown by greater N gains, and again the non-utilization of the liberated creatine by the new tissue. In this connection Shaffer (6) observed the excretion of creatine in exophthalmic goiter which was improving rapidly and Foster (17) has noted the persistence of creatinuria in typhoid convalescence accompanied by N gains.

¹ The suppression of lactation by bandaging is never complete. There is always a small production of milk even though it may never be expressed from the breasts. The small amount of creatine may thus have arisen as a sequel to the processes initiating lactation as in the case of W-r.

SUMMARY.

High calory, high carbohydrate diets do not check the normal involution of the human uterus. The N loss and creatinuria of the puerperium are referable to the processes attending the onset of lactation rather than to the involution of the uterus, though the latter factor is not necessarily excluded.

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A STUDY OF THE INFLUENCE OF KIDNEY FUNCTION ON THE CONCENTRATION OF CERTAIN NON- PROTEIN SULFUR COMPOUNDS IN THE BLOOD.

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Several years ago it was reported that in some cases of clinical nephritis (1) and in severe experimental uranium nephritis in animals (2) the content of inorganic sulfates of the blood is increased. The analytical method used in these observations was somewhat crude, although the best available at the time, and as we are now possessed of more reliable technique it has appeared desirable to check these earlier observations on the inorganic sulfates, and at the same time to determine the effect produced by the reduction of kidney efficiency on the other non-protein sulfur compounds of blood, ethereal sulfates, and neutral sulfur, for the quantitative determination of which micro methods are now available.

For the production of experimental nephritis we have used uranium nitrate, and with the aid of this nephrotoxic agent we have carried out two series of experiments, in one of which a mild type of poisoning was induced, while in the second series we have by the use of larger doses produced more severe kidney lesions.

Dogs were used exclusively. Before the start of the experiment several urine specimens were obtained from each animal and examined for the presence of albumin and casts. With one exception (Dog 9) we used only subjects whose urine gave negative tests for these bodies. During the experimental period the animals were confined in metabolism cages and the urine was collected in 24 hour amounts. The dogs were given water and food in quantities which were always in excess of their needs. The

food throughout the experiment remained approximately the same and consisted of a mixture of bread, sweet potatoes, meat, and a generous supply of cooked chicken bones.

The experiments were each divided into several periods of from 1 to 4 days in length, the samples of blood being invariably taken on the last day. The urines collected daily during each period were mixed so that the figures given represent the urine volume of the average 24 hour excretion for the period.

The blood specimens were collected by heart puncture and were analyzed for non-protein nitrogen by the method of Folin and Wu (3) and for inorganic and ethereal sulfates and neutral sulfur by the technique described by Denis and Reed (4).

Experiment 1 — Dog 1, female, weight 8.6 kilos Received 0.4 mg of uranium nitrate per kilo of body weight on May 24 On May 26 it was noted that the urine contained a large trace of albumin The excretion of this substance continued until the end of the experiment The animal continued to eat as usual during the entire period of observation (See Table I.)

TABLE I

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 23-24	33	300	0.95	1.15	2.1	5.36	7.46
" 24-26	35	570	0.80	0.2	1.0	4.60	5.60
" 26-29	31	1010	±0.03				6.00

TABLE II

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood.				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 23-24	28	350	0.91	1.94	2.85	4.52	7.37
" 24-26	32	330	0.90	1.32	2.22	4.34	6.56
" 26-29	29	1050	Low	Low	Low		5.07

Experiment 2—Dog 2, female; weight 7 kilos. Received 0.4 mg. of uranium nitrate per kilo of body weight on May 24. Albumin was first found in the urine on May 26. The animal continued to eat as usual during the entire period of observation. The blood sample taken at the end of the third period had such a small concentration of inorganic and ethereal sulfate and total sulfur that it was impossible to obtain even approximate readings in the nephelometer. (See Table II.)

Experiment 3—Dog 3, female; weight 6 kilos. Received 0.6 mg. of uranium nitrate per kilo of body weight on May 28. Albumin appeared in the urine 24 hours later. The animal continued to eat as usual during the experiment. In the samples of blood taken at the end of the second, third, and fourth periods the concentration of inorganic and total sulfates was so small that it was impossible to obtain even approximate readings in the nephelometer. In Table III the concentrations of these fractions have simply been designated as low. The same notation will be used in the succeeding tables.

TABLE III

Date	Blood non-pro- tein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 28-30	20	100	2 40	0 68	3 08	4 46	7 54
“ 30-June 1	37	500	Low.	Low	Low.		
June 1-3	33	1000	“	“	“		4 0
“ 3-6	32	610	“	“	“		4 2
“ 6-8	46	250	1 13	0 64	1 77	2 89	4 66
“ 8-9	49	250	1 60	0 60	2 20	2 90	5 1

TABLE IV

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 27-28	29	500	1 8	0 53	2 33	6 07	8 40
" 28-31	37	1000	1 33	0 01	1 34	6 79	8 13
May 31-June 2	35	1200	Low.	Low.	Low.		5 4
June 2-4	28	2000	"	"	"		6 0
" 4-7	51	1400	"	"	"		6 6
" 7-9	44	1000	"	"	1 20	5 80	7 0

Experiment 4—Dog 4, male; weight 19 kilos Received 0.6 mg of uranium nitrate per kilo of body weight on May 28, and a second dose of like size on June 4 Albumin appeared in the urine on May 29 and was excreted during the remainder of the experiment Food was eaten as usual. (See Table IV.)

Experiment 5.—Dog 5, female; weight 17 kilos Received 3 mg. of uranium nitrate per kilo of body weight on June 1 Albumin was found in the urine on June 2 Food was eaten as usual throughout the experiment In the samples of blood taken at the end of the second, third, and fourth periods the concentrations of inorganic and ethereal sulfates were so low that quantitative determinations could not be made (See Table V)

TABLE V

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 30-June 1	29	500	1.8	1.1	2.9	4.56	7.46
June 1-2	30	1000	Low	Low	Low		4.64
" 2-4	52	2000	"	"	"		6.04
" 4-7	75	2000	"	"	"		4.80
" 7-9	71	1000	0.67	1.37	2.04	5.50	7.54

Experiment 6—Dog 6, female, weight 15 kilos Received 3 mg of uranium nitrate per kilo of body weight on June 1 Albumin appeared in the urine on June 2 Food was eaten as usual throughout the experiment (See Table VI)

TABLE VI

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
May 30-June 1	28	1000	1.10	1.35	2.45	6.35	8.80
June 1-2	43	2000	Low		Low		7.77
" 2-4	88	1000	0.80	1.52	2.32	5.08	7.40
" 4-7	118	1000	0.83	1.39	2.22	5.55	7.77
" 7-9	75	1000	1.10	1.56	2.66	6.86	9.52

Experiment 7—Dog 7, female, weight 6 kilos Received 13.5 mg of uranium nitrate per kilo of body weight on Oct. 25 Urine contained albumin on Oct. 26, on Oct. 29 contained also many granular casts and red

blood cells. The animal started to vomit on Oct. 28 and continued to do so at intervals until death. No food was eaten after Nov. 5. The animal was found dead in the cage on Nov. 7. (See Table VII)

TABLE VII

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
Oct 27-28	30	330	3 12	1 16	4 28	4 59	8 87
" 28-29	110	320	3 81	0 66	4 47	4 97	9 44
" 29-Nov. 1	242	220	7 02	2 23	9 25	4 15	13 4
Nov 1-7	395	210	8 32	8 68	17 00	5 9	22 9

Experiment 8.—Dog 8, female; weight 7 5 kilos. Received 13 5 mg of uranium nitrate per kilo of body weight on Oct 25. Albumin appeared in urine on Oct 26, and in addition coarsely granular casts on Oct 27. On Oct 31 the animal started to vomit and continued to do so at intervals during the remainder of the experiment. No food was eaten after Oct 28 (See Table VIII)

TABLE VIII

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
Oct 24-25	39	350	3 00	0 88	3 88	4 53	8 41
" 25-29	89	570	2 30	0 25	2 55	5 75	8 30
" 29-Nov 1	249	130	7 84	3 77	11 61	3 84	15 45
Nov 1-3	399	0	10 29	4 91	15 2	4 90	20 1

TABLE IX

Date	Blood non-protein nitrogen	Average volume of 24 hr urine	Mg S per 100 cc blood				
			Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
1926	mg per cent	cc					
Nov 14-15	25	900	4 86	1 26	6 12	5 48	11 6
" 15-18	96	1683	4 06	0 80	4 86	5 74	10 6
" 18-20	222	268	10 33	1 07	11 4	7 10	18 5

Experiment 9 — Dog 9, female; weight 8.2 kilos. The urine for 6 days showed a small trace of albumin but no casts. 13.5 mg. of uranium nitrate per kilo of body weight were injected on Nov. 15. On Nov. 16 the urine showed a very large trace of albumin and many granular casts. Food was refused on and after Nov. 17. (See Table IX.)

The experiments presented above confirm to a certain extent the results published some years ago but they also bring out several points which were not noted in this earlier work.

In Experiments 1 and 2 extremely small doses of uranium were given. That the dosage was sufficiently large to produce a pathological condition of the kidney is shown however by the prompt appearance and persistence of an unmistakable albuminuria. There was however no retention of non-protein nitrogen in the blood, whereas the concentrations of the non-protein sulfur compounds (inorganic and ethereal sulfates and neutral sulfur) were reduced. The diuresis which has been noted by many investigators (5) as a more or less constant phenomenon in early uranium poisoning was also observed in these experiments.

In Experiments 3 and 4, the dosage of uranium nitrate was increased to 0.6 mg. per kilo of body weight. The administration of this amount was followed by marked albuminuria and diuresis, a moderate retention of non-protein nitrogen, and a marked reduction of the concentration of the non-protein sulfur compounds of the blood.

In Experiments 5 and 6 the amount of uranium nitrate was again increased (to 3 mg. per kilo) with the production of results somewhat similar to, but more striking than those obtained with the two animals used in the preceding experiments. For Dog 5, in which diuresis was severe, low values for the non-protein sulfur compounds were observed in three blood samples taken over a period of 7 days. In the last observation made 9 days after the injection there appeared to be some indication that these bodies were beginning to increase although the amounts noted were still below the initial level. For Dog 6 low values for the sulfur bodies were obtained on one observation only, while the analyses of subsequent samples showed a gradual rise in all the sulfur fractions. In the results of these two experiments the entire lack of concordance between the retention of non-protein nitrogen and of the non-protein sulfur bodies is unmistakable.

In the three last experiments (Nos. 7, 8, and 9) the enormous dosage of 13.5 mg. of uranium nitrate per kilo of body weight was given. In these animals there was noted a large and prompt rise in the non-protein nitrogen of the blood. In one case (Dog 7) the rise in the inorganic sulfates appeared to coincide with the retention of nitrogen bodies, and in this animal no period of diuresis was noted. In the other two subjects (Dogs 8 and 9) while there was a prompt elevation of the non-protein nitrogen there was noted an unmistakable fall in the inorganic sulfates in the samples of blood taken 3 to 4 days after the injections, whereas the analyses of samples taken later showed a large increase in all of the sulfur compounds for which figures are available. In these two animals there was a considerable increase in the output of urine following the administration of the drug, whereas during the last 2 days of the experiment the amount was much reduced, and in one case (Dog 8) complete anuria was present. It is to be regretted that the analytical method used was not sufficiently sensitive to allow us to measure the amount of sulfates in the blood when these bodies are present in the extremely high dilutions noted in some of our experiments. We have found it easily possible to read a suspension of barium sulfate representing a concentration of 0.02 mg. of S in 25 cc. Greater dilutions cannot be read with any degree of accuracy. On the basis of the dilution employed (4) we have therefore calculated that where we have placed the notation of "low" in the tables of results it would indicate that the maximum concentration of sulfur present per 100 cc. of blood would amount to 0.5 mg. for inorganic and total sulfates and 1.6 mg. for total sulfur. The values for total sulfur, which in some of the experiments show marked reduction, do not as a whole show the same degree of diminution as do the figures obtained for inorganic and for total sulfates.

The fact has been suggested to us that there may be present in the blood of the animals that show extremely low values for inorganic and total sulfates some substance which interferes with the precipitation of barium sulfate.

In order to throw light on this point we have carried out the following experiment.

A dog to which we administered 0.6 mg. of uranium nitrate per

kilo of body weight gave the following figures on analysis of blood taken before the injection:

Non-protein nitrogen . . .	35	mg	per 100 cc. of blood.
Total sulfates (as S) . . .	3 27	" "	100 " " "
" sulfur . . .	8 30	" "	100 " " "

The animal showed the usual diuresis with 24 hour urine volumes of approximately 2000 cc. and 5 days later the following results were obtained:

Non-protein nitrogen	39	mg	per 100 cc of blood
Total sulfates (as S), too low to read			
" sulfur	6 37	mg	per 100 cc of blood.

To 10 cc. of the trichloroacetic acid filtrate (which gave a precipitate for total sulfates of such small concentration that it could not be read in the nephelometer) there was added 0.1 mg. of S (as K_2SO_4). When read in the nephelometer against a suitable standard this gave a value of 0 101 mg of S.

To a 10 cc portion of the same blood there was added 0 5 mg. of S (as K_2SO_4). After precipitation this sample gave when subjected to the usual analytical procedure a total sulfate of 4.98 mg. of S per 100 cc. of blood. A 10 cc portion of blood (which also gave values for total sulfates which were too low to read, and a non-protein nitrogen of 57 mg) taken 2 days later from the same animal, gave on the addition of 0 5 mg. of S (as K_2SO_4) 5 03 mg. of total sulfates per 100 cc of blood

It has occurred to us that the protein content of the blood of our experimental animals might be much reduced, and that if so our trichloroacetic acid filtrates might be more acid than is usually the case, a fact which might interfere with the precipitation of barium sulfate in our method for inorganic sulfates, although it would have no effect in the case of the total sulfates or total sulfur.

The following determinations of the plasma proteins of this dog made by the methods of Wu (6) indicate that the reduction in these cases is so slight that it cannot be considered to have any influence on the result.

	Sample 1.	Sample 2 (taken 5 days after Sample 1)	Sample 3 (taken 2 days after Sample 2)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Albumin	3 9	3 3	3 7
Globulin	1 8	1 8	1 8
Fibrin	0 35	0 56	0 46

The experimental results presented above show in our opinion, that there is no substance present in the blood of animals suffering from uranium nephritis which interferes with the precipitation of barium sulfate under the conditions of the analytical method employed.

The results obtained in this series of animals indicate that the degree of retention of the non-protein nitrogen and of the non-protein sulfur bodies of the blood bears no direct relation in the case of animals whose kidney function has been reduced by uranium poisoning.

According to Cushny (7), urea (which is responsible for at least 50 per cent of the non-protein nitrogen of the blood) "stands midway between the chlorides with a definite threshold, and some other substances which are believed to be excreted to the utmost degree," whereas for sulfates the same author states, that, "There is no evidence that they are retained in the plasma when the supply is reduced, and if there is any threshold for the sulfates it must be so low that it is beyond the present methods employed in their estimation."

Our results show in every case the unmistakable effect of increase in urine output on the concentration of the non-protein sulfur compounds of the blood, for which diuresis there was invariably noted a distinct fall in the concentration of the inorganic and ethereal sulfates and neutral sulfur, a fall which was frequently accompanied by a marked increase in the concentration of non-protein nitrogen. In the cases of more severe poisoning where the output of urine was diminished there was noted however a rise, in some cases of considerable magnitude, in the concentration of the inorganic sulfates, with a less striking increase in the neutral sulfur. In some cases the ethereal sulfates also showed increased values whereas in others this was not observed. As regards the

relative concentration of non-protein sulfur bodies it was found in the majority of cases that during the period of high urinary output the per cent of total sulfur present as total sulfate was decreased while the neutral sulfur percentage increased, whereas when the volume of urine was diminished the reverse was noted.

SUMMARY.

In dogs poisoned with uranium nitrate it was found that the concentration of the inorganic sulfates and of the neutral sulfur fraction of the blood was directly dependent on the volume of urine excreted, and that in the case of animals with diuresis whose kidneys had been injured to such an extent that the non-protein nitrogen of the blood was much increased, there was frequently found a large decrease in the inorganic and total sulfates, and a less striking fall in the neutral sulfur fraction. As the volume of urine diminished to normal or subnormal values the concentration of inorganic sulfate and of neutral sulfur slowly rose, and in some cases attained values from 100 to 300 per cent above the normal concentration.

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CONCERNING THE EFFECT PRODUCED BY THE ADMINISTRATION OF SULFUR ON THE CONCENTRATION OF CERTAIN SULFUR COMPOUNDS IN BLOOD AND URINE.*

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According to the views most generally held at present the various types of sulfur compounds found in urine, *i.e.* inorganic and ethereal sulfates, and neutral sulfur arise from the metabolism of protein and sulfolipoids in the cells. What proportion of any of these bodies is formed from the hydrogen sulfide invariably present in the large intestine is not known. The fact has long been recognized that when powdered sulfur is taken by mouth, it is, although practically insoluble in water, attacked by the bacteria present in the lower part of the alimentary canal, with the production of large amounts of hydrogen sulfide. This phenomenon has furnished a means whereby it would appear possible to determine approximately the relative importance of intracellular metabolism, and of bacterial action in the intestine in connection with the formation of the sulfur-containing bodies of the blood and urine.

EXPERIMENTAL METHODS.

Determinations of the inorganic and ethereal sulfates and neutral sulfur have been made in the blood by means of the methods of Denis and Reed (1) and in urine by the procedures of Folin (2) and of Benedict (3).

Our experiments have been carried out on dogs and on one goat. In the case of the goat the animal was allowed to

* Assisted by a grant from the David Trautman Schwartz Research Fund.

graze during the day and was also given daily a small portion of oats. It was not possible to collect the urine in this case so that only the data on blood are available. After a preliminary sample (50 cc.) of blood was taken the animal was given by mouth 10 gm. of powdered sulfur for 5 days, and 20 gm. for 3 days, making a total of 110 gm. of sulfur administered. Blood was taken from the

TABLE I

Goat, female; weight 30.5 kilos. During Period B 10 gm. of powdered sulfur were given by mouth for 5 days, and 20 gm. for the final 3 days.

Period	Duration	Blood Mg S per 100 cc				
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	<i>days</i>					
A	8	6.5	0.65	7.15	5.67	12.82
B	7	10.9	2.97	13.87	6.73	20.60

TABLE II

Dog 1, female, weight 10.9 to 12.5 kilos. Food, 17 gm. of Cowgill's casein vitamin B mixture per kilo per day. During Period B, 0.5 gm. of sulfur per kilo per day.

Period	Duration	Blood Mg S per 100 cc					Urine					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	days						cc					
A	5	2 44	0 79	3 23	5 30	8 53	680	0 28	0 08	0 36	0 02	0 38
B	5	3 06	0 94	4 00	6 68	10 68	646	0 70	0 17	0 87	0 01	0 88
C	4	3 53	1 16	4 68	5 72	10 40	645	0 22	0 12	0 34	0 02	0 36

jugular vein on the morning of the 9th day, 18 hours after the administration of the last dose of sulfur.

The results of this experiment given in Table I indicate that after the administration of sulfur the content of the blood in inorganic and ethereal sulfates was considerably increased, while the neutral sulfur fraction also showed a rise in value. The largest proportional rise was shown by the ethereal sulfate fraction,

while there was a distinct fall (from 44.5 to 32.6 per cent of total sulfur) in the proportionate value for neutral sulfur.

Further experiments were carried on with dogs. These animals were kept in metabolism cages and were fed during a preliminary period of 4 days and during three subsequent periods a weighed

TABLE III

Dog 2, female; weight 14.5 to 13.6 kilos Food, 17 gm of Cowgill's casein vitamin B mixture per kilo per day During Period B, 0.5 gm. of sulfur per kilo per day.

Period	Duration	Blood Mg S per 100 cc					Urine					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	days						cc					
A	5	2 64	0 36	3 00	4 31	7 31	410	0 36	0 05	0 41	0 17	0 58
B	5	2 96	0 65	3 61	5 78	9 39	536	0 79	0 06	0 85	0 19	1 04
C	3	2 50	0 72	3 22	6 55	9 77	317	0 47	0 02	0 49	0 16	0 65

TABLE IV.

Dog 3, female; weight 7.0 kilos Food, 300 gm of raw beef, 7 gm. of fullers' earth During Period B, 0.5 gm of sulfur per kilo per day.

Period	Duration	Blood Mg S per 100 cc					Urine					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	<i>days</i>						<i>cc</i>					
A	6	2 75	1 15	3 9	5 8	9 7	211	0 23	0 14	0 37	0 07	0 44
B	4	2 56	0 75	3 31	6 6	9 91	229	0 70	0 17	0 87	0 12	0 99

ration of the mixture of caseins, lard, etc., described by Cowgill (4), or in some cases on raw lean beef, or bread and milk. The urine was collected daily by catheter and was preserved with chloroform. At the end of each period all the urines collected from each animal were mixed and the analyses made on this com-

TABLE V

Dog 4, female; weight 10 kilos. Food, 400 gm of raw lean beef, 7 gm of fullers' earth During Period B, 0.5 gm of S per kilo per day.

Period	Duration	Blood Mg S per 100 cc					Urine.					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm. S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	<i>days</i>						<i>cc</i>					
A	6	3.52	0.58	4.10	6.03	10.13	232	0.28	0.08	0.36	0.09	0.45
B	4	2.95	0.52	3.47	7.38	10.85	238	0.83	0.12	0.95	0.15	1.10

TABLE VI

Dog 5, female, weight 7.5 kilos Food, 150 gm of bread, 200 cc of milk, 9 gm of fullers' earth During Period B, 0.5 gm of sulfur per kilo per day

Period	Duration	Blood Mg S per 100 cc.					Urine					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	<i>days</i>						<i>cc</i>					
A	5	1.72	0.85	2.57	6.24	8.81	93	0.04	0.004	0.044	0.01	0.054
B	5	5.12	3.48	8.60	8.7	17.3	97	0.25	0.010	0.26	0.14	0.40

TABLE VII

Dog 6, female, weight 10 kilos Food, 250 gm of bread, 250 cc of milk, 9 gm of fullers' earth During Period B, 0.5 gm of sulfur per kilo per day

Period	Duration	Blood Mg S per 100 cc					Urine					
		Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S	Average volume	Gm. S in 24 hrs				
								Inorganic SO ₄	Ethereal SO ₄	Total SO ₄	Neutral S	Total S
	<i>days</i>						<i>cc</i>					
A	5	2.06	0.60	2.66	7.99	10.65	377	0.17	0.02	0.19	0.05	0.24
B	5	2.40	0.60	3.00	8.27	11.27	349	0.39	0.03	0.42	0.08	0.50

posite sample. The total volumes were then divided by the number of days in the period in order to obtain an average volume for a 24 hour excretion. Samples of blood of approximately 20 cc. volume were taken from the heart at the end of each experimental period.

During the first period (A) no sulfur was given, during the second (B) 0.5 gm. of powdered sulfur per kilo of body weight was administered daily, well mixed with the food, while during the third period (C) the administration of sulfur was again omitted. The results obtained on several experiments of this type are collected in Tables II to VII inclusive.

The experiments on dogs described above have given some rather unexpected results. As regards the urine figures there is found an absolute increase in all of the various fractions of sulfur-containing bodies; the point of chief interest lies however in the fact that the increase in total sulfur (which would give an indication of the amount of ingested sulfur absorbed) is so small,—the increase for the six animals used averaging only 0.5 gm., a quantity which in most cases is approximately 10 per cent of the quantity of sulfur administered. The continued increased excretion in the third period (during which no sulfur was given) may best be explained by the assumption that this is due to a residue of sulfur remaining in the intestine after the administration of this substance had been discontinued. In connection with this statement it is of interest to note that these animals did not show any evidence of diarrhea which frequently follows the ingestion of sulfur in human beings, which was probably due to the fact that they received a daily dose of fullers' earth.

The level of this increase in total urinary sulfur (which amounts to from 100 to 700 per cent) varies greatly in the different animals, and is apparently independent of the type of diet used. As sulfur is insoluble in water and also in the slightly alkaline intestinal contents, it seems probable that this increase in total urinary sulfur must be due to the oxidation of hydrogen sulfide absorbed from the intestine.

As regards changes in the individual sulfur fractions of the urine it is evident that the chief increase occurs in the inorganic sulfate, although in some cases (Dogs 1 and 5) there is noted a

considerable elevation of the ethereal sulfate fraction, and in all the experiments a rise in neutral sulfur.

The results obtained on the blood are not so uniform as in urine. In the case of the goat there was a marked absolute increase in all the sulfur compounds, and a relative increase in the ethereal sulfate fraction. In the case of the dogs there appears to be a good deal of variation between individual experiments; in two cases, Dogs 3 and 4, there was noted an absolute decrease in the content of inorganic and ethereal sulfates, the slight rise in total sulfur being accounted for by an increase in the neutral sulfur fraction. These animals were fed on raw beef. In the case of Dog 6 (fed on bread and milk) there was no change in either the inorganic or ethereal sulfates of the blood although in the urine both of these fractions showed a considerable increase. In the remaining three experiments (Dogs 1, 2, and 5) there was obtained an unmistakable rise in all of the non-protein sulfur fractions of the blood, although there is a considerable variation in the magnitude of the increase.

So little experimental work on the non-protein sulfur compounds of blood has been carried on, and our knowledge regarding the conditions which influence the concentration of these bodies is so limited, that speculation regarding this matter would appear futile at present.

SUMMARY.

Examination of the urine of animals that had been fed a standard ration to which had been added powdered sulfur to the extent of 0.5 gm. per kilo per day showed that an average of only 10 per cent of the administered sulfur had been absorbed. As sulfur is insoluble in water or in dilute sodium bicarbonate solution of the alkalinity of intestinal contents (pH 7.0 to 8.0) it is assumed that the observed increases in total urinary sulfur are due to the oxidation of hydrogen sulfide formed in the intestine by bacterial action on the sulfur. As these increases in total urinary sulfur amounted in some experiments to several hundred per cent of the basal value it would appear probable that a portion of the sulfur-containing bodies of the urine may not originate exclusively from intracellular metabolism of protein fragments but may arise in part from the oxidation of the intestinal hydrogen sulfide.

Analysis of the blood showed in some cases an increase in the non-protein sulfur compounds and in others a marked decrease.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

X. THE INFLUENCE OF ULTRA-VIOLET LIGHT UPON CALCIUM AND PHOSPHORUS METABOLISM IN MILKING COWS.*

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In 1924 (1) we demonstrated with lactating goats under exposure to ultra-violet light for 20 minutes daily that calcium assimilation was favorably influenced and that negative calcium balances became positive under such exposure. These data were in harmony with what had been observed in the healing of rickets in man, the rat, and the chicken under exposure to ultra-violet light; although in the case of the rat it may still be a question whether the improved calcium assimilation under exposure to ultra-violet light is a direct effect or a secondary result of the consumption of activated excreta or skin secretions (2).

In 1925 (3) we studied the effect of direct sunlight upon the calcium assimilation in milking cows. The first part of this experiment was carried out in June when the ultra-violet intensity of the sunlight was at its maximum. In several earlier calcium and phosphorus balance experiments with cows the experiment had always been conducted in a basement and away from direct sunlight. As knowledge of the influence of the ultra-violet rays on calcium and phosphorus assimilation developed it became clear that in all probability we had not reckoned with the potent-factor

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of direct sunlight in these earlier experiments and consequently had limited our data in its application more particularly to winter feeding than to summer conditions.

In these experiments with milking cows and direct sunlight negative balances continued during the sunlight period although the degree of negativity was somewhat reduced. Reasoning from experiments conducted with goats under the influence of ultra-violet light, we were led to say that "Apparently summer sunlight in comparison with the radiations of a quartz mercury vapor lamp is feeble in its antirachitic properties when considered in relation to liberally milking animals." We did not know at that time that there was a difference between species—particularly between goats and cows—in their susceptibility to direct irradiation with ultra-violet light. That such a difference exists in respect to calcium assimilation is evident from the data to be presented in this paper.

Because our earlier experiments on the influence of June sunlight on calcium assimilation in milking cows seemed to show slight improvement in calcium assimilation during the sunlight period, it was decided to repeat the work but to intensify the possible action through the use of ultra-violet light from quartz mercury lamps.¹

EXPERIMENTAL.

On September 3, 1926, three pure bred Holstein cows were selected for this experiment. From that date until the beginning of the metabolism experiment on October 26 they were kept indoors. Previous to September 3 they had been on pasture and in addition received some alfalfa hay and grain. After September 3 and until October 11 they received a ration of grains, corn silage, and alfalfa hay. From October 11 until the beginning of the actual metabolism trial and throughout the trial itself, the daily ration consisted of 30 pounds of corn silage, 5 pounds of alfalfa hay of unknown curing history, 5 pounds of timothy hay of unknown curing history, and 1 pound of a grain mixture consisting of 59 parts of yellow corn, 25 parts of wheat bran, 15 parts

¹ The lamps used in this work were kindly loaned us by the Hanovia Chemical and Manufacturing Company and the Cooper Hewitt Electric Company.

of oil meal, and 1 part of common salt, for every 3 to 4 pounds of milk produced.

These cows were yielding a generous amount of milk varying from 35 to 55 pounds per day, and it was believed that a mixture of equal parts of alfalfa hay and timothy hay would place the calcium intake at such a level as to force all the cows into negative calcium balances. In studies of this kind it was desirable to have the animals in negative lime balance especially during the preultra-violet light period, because if there is improved calcium assimilation under the influence of any factor being studied it would be shown by the double index of reduced fecal calcium as well as a movement toward the positive in the calcium balances. Of course, with constant milk secretion the latter will depend upon the former.

Cow 1 was 8 years old, fresh August 29, 1926, and was not bred at the time of the experiment. The back was covered with short white hair with no black spots in the area irradiated.

Cow 2 was 13 years old. It was fresh June 16, 1926, and was not bred at the time of the experiment. The back was also covered with white hair and with no black spots in the area irradiated.

Cow 3 was 11 years old. It was fresh August 27, 1926, and was not bred at the time of the experiment. The back was covered mainly with black hair with an occasional spot of white.

The metabolism experiment was divided into two parts; first, a preliminary period of 3 weeks in which the animals received no ultra-violet light, the experiment being conducted in a basement room with closed windows and only ordinary electric bulbs as sources of needed light; second, a period of 4 weeks in which the animals were irradiated 1 hour daily by a quartz mercury vapor lamp. The irradiation was conducted at a distance of 22 inches over the back of the animal but well toward the hips and while the animal was standing. During the period of irradiation the mangers were covered with removable beaver board screens in order to prevent any activation of feed residues.

Quantitative collection of the excreta was made with daily sampling for analysis. The milk was composited for 7 day periods for analysis. During the entire metabolism period the cows were quiet and contented and the daily ration was completely consumed. Cow 1 weighed 1392 pounds at the beginning of the

metabolism experiment and 1306 at the end; Cow 2 weighed 1253 pounds at the beginning of the experiment and 1244 pounds at its termination; Cow 3 weighed 1330 pounds at the beginning and

TABLE I
Record of Calcium Balance and Milk Production of Animal 1

Period	CaO in feces	CaO in milk	CaO in urine	Total CaO excreted	Total CaO intake	Balance per wk	Milk per wk
Preultra-violet light period							
	gm	gm	gm	gm	gm	gm	lbs
Oct. 25–Nov 1	598 51	289 04	12 00	899 55	707 09	–192 40	358
Nov. 1–8	503 47	251 89	10 03	765 39	762 47	–2 92	334
“ 8–15	555 30	234 75	12 74	802 79	804 02	+1 23	331
Ultra-violet light period—1 hr daily							
Nov 15–22	554 11	282 72	11 64	848 47	804 02	–44 45	343
“ 22–29	555 21	275 86	11 40	842 47	807 83	–35 64	330
“ 29–Dec 6	627 91	299 00	10 41	937 32	807 83	–129 49	329
Dec. 6–13.	559 76	270 94	15 09	845 79	807 83	–37 96	324

Record of Phosphorus Balance of Animal 1

Period	P ₂ O ₅ in feces	P ₂ O ₅ in milk	P ₂ O ₅ in urine	Total P ₂ O ₅ excreted	Total P ₂ O ₅ intake	Balance per wk
Preultra-violet light period						
	gm	gm	gm	gm	gm	gm
Oct. 25–Nov. 1	776 44	323 14	16 89	1116 48	944 84	–171 64
Nov. 1–8	655 25	309 55	5 75	970 55	955 73	–14 82
“ 8–15	695 97	272 37	14 21	982 55	970 26	–12 29
Ultra-violet light period—1 hr daily						
Nov. 15–22	625 03	323 22	11 00	959 25	970 26	+11 01
“ 22–29	673 41	293 85	3 29	970 55	943 08	–27 47
“ 29–Dec 6	682 17	310 96	3 92	997 05	943 08	–53 97
Dec 6–13	678 02	294 50	3 61	976 13	943 08	–33 05

1254 at the end. Cow 3 gave us an incomplete 7 weeks record because toward the end of the 5th week and after nearly 2 weeks of irradiation, it died suddenly. It had been in apparently normal

health, had consumed the daily ration completely, and had given a normal amount of milk on the day of its sudden death. Death

TABLE II
Record of Calcium Balance and Milk Production of Animal 2

Period	CaO in feces	CaO in milk	CaO in urine	Total CaO excreted	Total CaO intake	Balance per wk	Milk per wk
Preultra-violet light period.							
	gm	gm	gm	gm	gm	gm	lbs
Oct. 25–Nov 1	419 79	214 55	9 70	644 04	707 09	+63 05	291
Nov 1–8	479 55	200 50	15 67	695 72	762 47	+66 75	283
“ 8–15	488 40	179 45	13 53	681 38	781 13	+99 75	261

Ultra-violet light period—1 hr daily							
Nov 15–22	540 96	209 43	10 47	760 86	781 13	+20 27	250
“ 22–29	541 44	204 11	10 95	756 50	784 94	+28 44	249
“ 29–Dec 6	530 10	204 49	10 31	744 90	784 94	+40 04	239
Dec 6–13	583 51	204 07	19 06	806 64	784 94	–21 70	248

Record of Phosphorus Balance of Animal 2.

Period	P ₂ O ₅ in feces	P ₂ O ₅ in milk	P ₂ O ₅ in urine	Total P ₂ O ₅ excreted	Total P ₂ O ₅ intake	Balance per wk
Preultra-violet light period						
	gm	gm	gm	gm	gm	gm
Oct 25–Nov. 1	592 02	243 69	9 70	845 41	944 84	+99 43
Nov. 1–8	625 51	254 48	8 64	888 63	955 73	+67 10
“ 8–15	564 30	232 93	7 60	804 83	883 81	+78 98

Ultra-violet light period—1 hr. daily.						
Nov 15–22	631 68	223 09	8 93	863 70	883 81	+25 11
“ 22–29	595 58	226 79	3 58	825 95	856 63	+30 68
“ 29–Dec 6	615 60	213 19	8 70	837 49	856 63	+19 14
Dec. 6–13	597 74	220 98	5 23	823 95	856 63	+32 68

was due to a hemorrhage occasioned by the puncture of the vena cava by a foreign body. We consider the record so far as it went as a normal one and are recording it as such. The calcium and

phosphorus balance sheets of the animals are shown in Tables I to III.

TABLE III
Record of Calcium Balance and Milk Production of Animal 3

Period	CaO in feces	CaO in milk	CaO in urine	Total CaO excreted	Total CaO intake	Balance per wk	Milk per wk
Preultra-violet light period							
	gm	gm	gm	gm	gm	gm	lbs
Oct 25–Nov. 1	456 48	276 22	19 88	752 58	707 09	−45 49	368
Nov. 1–8	466 24	280 45	11 94	758 93	762 47	+3 54	374
“ 8–15	549 81	245 10	20 99	815 90	804 02	−11 88	359
Ultra-violet light period—1 hr daily							
Nov. 15–22	482 40	314 42	16 44	813 26	804 02	−9 24	346
“ 22–27*	405 75	210 31	10 64	626 70	587 02	−39 68	263
Died suddenly Nov 28							

Record of Phosphorus Balance of Animal 3

Period	P ₂ O ₅ in feces	P ₂ O ₅ in milk	P ₂ O ₅ in urine	Total P ₂ O ₅ excreted	Total P ₂ O ₅ intake	Balance per wk
Preultra-violet light period						
	gm	gm	gm	gm	gm	gm
Oct. 25–Nov. 1	415 15	297 98	9 66	1022 79	944 84	−77 95
Nov 1–8	611 20	338 60	6 14	955 94	955 73	−0 21
“ 8–15	575 64	312 09	9 51	897 24	970 26	+73 02
Ultra-violet light period—1 hr daily						
Nov 15–22	576 00	320 71	9 47	906 18	970 26	+64 08
“ 22–27*	456 14	239 47	4 50	700 11	673 59	−26 52

* 5 day period.

We had expected negative calcium balances with all the animals, especially in the preultra-violet light period. But in the case of Cow 2, whose milk production was about 35 pounds of milk daily but whose calcium intake was similar to that of the other two animals, a positive balance prevailed in the preultra-violet light

period and throughout the ultra-violet period with the exception of the last week when the records show a negative calcium balance.

We had expected positive phosphorus balances or equilibrium in the phosphorus balance sheet with all the animals because of the liberal provision of this element in the ration. But with Animal 1 a negative phosphorus balance, although not of high order, prevailed throughout the experiment with the exception of 1 week. In the case of the other two animals either positive phosphorus balances or equilibrium prevailed as in the case of Cow 3.

DISCUSSION.

From a study of the data secured one is forced to the conclusion that calcium and phosphorus assimilation has not been improved in this species by an intensified exposure to ultra-violet light. In the case of Animal 1 a negative lime balance prevailed in the preultra-violet light period and continued throughout the entire period of irradiation. There was no reduction in the loss of calcium from the fecal residues in the ultra-violet period as compared with the preultra-violet light period, and in studies of this character this is always an excellent index of a positive effect of the antirachitic factor. If one wished to interpret the figures rigidly, the data would actually indicate a somewhat less absorption of calcium during the ultra-violet light period as compared with the preultra-violet light period. However, no such close interpretation should be made. All that can be said from the record of Animal 1 is that the effect on calcium assimilation of exposure to ultra-violet light was negative.

The phosphorus balances with this animal were all negative with the exception of 1 week. It has been repeatedly observed in our metabolism work with cows that when the plane of intake of phosphorus is liberal as can be secured through the introduction of 20 to 25 per cent of wheat bran in the grain part of the ration, equilibrium or positive phosphorus balances will follow. The record of this animal is an exception to what we have usually observed on intakes of 900 to 1000 gm. weekly of P_2O_5 with the cow milking 50 pounds daily. In the case of Animal 1 there was not a reduction of the fecal phosphorus during the ultra-violet light period as compared with the preultra-violet light period,

indicating as in the case of calcium that the exposure to ultra-violet light had had little, if any, influence on calcium and phosphorus metabolism.

The milk flow of the animal was well sustained throughout the experimental period, but there was no increase in milk flow during the ultra-violet light exposure. There was no such response in production under exposure to ultra-violet light as has been observed in egg production in the case of laying hens, which had previously been depleted of the antirachitic factor.

Further, there was no increase during the ultra-violet period in the calcium secreted into the milk. In fact we have not observed this even in the case of goats which do respond in improved calcium assimilation to direct irradiation with ultra-violet light.

The record of Animal 2 so far as improvement in calcium and phosphorus assimilation by irradiation is concerned was a duplication of the results secured with Animal 1. With lowered milk production (35 pounds 'per day) this individual remained in positive calcium and phosphorus balance during the entire experimental period with the exception of the last week of the irradiation period when the calcium balance became negative. There seemed to be an increased loss of calcium in the feces during the irradiation period rather than a decreased loss, although we have no reason whatever to believe that the ultra-violet light acted adversely on calcium assimilation. We have no explanation to offer for this result. Certainly, as in the case of Animal 1, this animal was not benefited in its calcium and phosphorus assimilation by exposure to ultra-violet light. Further, there was no response to ultra-violet light in increased milk production, although the level of production was maintained on practically the same plane throughout the entire metabolism experiment, including both the preirradiation and irradiation periods. The total calcium secreted into the milk remained very constant throughout the entire experiment and was not increased through irradiation.

Animal 3 gave results, so far as the influence of ultra-violet light is concerned, similar to those secured with the other two animals. During the preirradiation period this animal was in negative calcium balance although for 1 week a slight positive balance of 3.5 gm. was recorded. During the irradiation period, which in this case lasted only 12 days because of sudden

death, the negative calcium balance continued. Intestinal absorption of calcium was not improved—the calcium losses in the feces being quite as large as during the preirradiation period. With this animal phosphorus equilibrium prevailed throughout the experiment, the retention of phosphorus practically balancing the loss. There was no noticeable improvement in fecal absorption during the irradiation period. Milk secretion was sustained but not increased by irradiation, and the calcium content of the milk remained at the same level in both periods.

Apparently then from the data so far secured ultra-violet light is not, through direct impingement on the animal, a factor of consequence in the calcium and phosphorus metabolism of the dairy cow. The observation made in 1925 (3) that there was slight improvement in the calcium balances of milking cows during the sunlight period, taken as a whole, as contrasted with the no sunlight period, must be explained on a basis other than that of a direct irradiation effect.

It is possible that in the case of the cow ultra-violet light is ineffective because of lack of penetration of the skin. It is recognized that the skin of the cow is considerably thicker than that of the goat, but failure of the occurrence and resorption of skin secretions which could be activated by direct irradiation is an alternative which should be considered.

SUMMARY.

1. Apparently ultra-violet light has little, if any, direct influence upon the calcium and phosphorus metabolism of dairy cows.
2. Ultra-violet light has no influence, either favorable or adverse, upon the milk production of cows.
3. Ultra-violet light has no apparent influence upon the calcium and phosphorus content of the milk secreted.
4. It is suggested that this species (the cow) derives its anti-rachitic vitamin from the feed and is different in this respect from man, the goat, the chicken, and probably the rat, all of which can be favorably influenced directly by the short wave-lengths of solar radiation.
5. These data should not be interpreted as indicating that exposure of the dairy cow to sunlight is not beneficial.

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**A NOTE ON THE USE OF MAGNESIUM PERCHLORATE
TRIHYDRATE (DEHYDRITE) AND ASBESTOS-SODIUM
HYDROXIDE (ASCARITE) IN GRAVIMETRIC METAB-
OLISM DETERMINATIONS, FOR WATER
AND CARBON DIOXIDE ABSORPTION.***

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Sulfuric acid and soda-lime or caustic soda are generally used as absorbents for water and carbon dioxide in gravimetric metabolism determinations. We have experimented with these and several other absorbents in a metabolism apparatus designed for small animals such as mice, rats, or guinea pigs. The train of tubes consisted of a tower containing a carbon dioxide absorbent; one containing a water absorbent; a tube containing clean pumice stone saturated with water, for moistening the air delivered to the animal; the animal chamber, two Blount gas absorption tubes in series, each containing a water absorbent; and two or three Blount tubes in series each containing on the one side a carbon dioxide absorbent and on the other a water absorbent. The gain or loss of each tube was determined by weighing it to 0.1 mg. The Blount tubes are especially suitable for such work because of their lightness and the fact that the water formed on the one side by the reaction of the carbon dioxide is absorbed on the other side of the same tube and hence weighed at the same time. We have used in this metabolism train sulfuric acid, phosphorus pentoxide, and magnesium perchlorate trihydrate as water absorbents, and soda-lime, crushed shell sodium hydroxide, and asbestos impregnated with sodium hydroxide as carbon dioxide absorbents.

* Both the $\text{Mg}(\text{ClO}_4)_2 \cdot 3\text{H}_2\text{O}$ and the asbestos-sodium hydroxide may be obtained from Arthur H. Thomas Co., Philadelphia. The first is sold under the trade name of dehydrate, and the last named material is sold under the trade name of ascarite.

The objections to the water absorbents hitherto used are numerous. The fact that sulfuric acid is a liquid often makes it undesirable to use. Pressure changes may send the liquid into other parts of the system and even into the soda-lime container unless a trap is used. Haldane (1) recommended the use of pumice stone freed of organic matter and saturated with sulfuric acid. This is perhaps the best manner of using the acid in metabolism work, but the digestion of the organic matter in the pumice stone is troublesome, and in use dilute sulfuric acid collects at the bottom of the mass. Furthermore there is no way of ascertaining the extent of the exhaustion of the absorbent. Phosphorus pentoxide is an efficient absorbent but becomes sticky and very difficult to manipulate.

Magnesium perchlorate trihydrate $\text{Mg}(\text{ClO}_4)_2 \cdot 3\text{H}_2\text{O}$ is an entirely satisfactory water absorbent from every standpoint. This material is sold under the trade name of dehydrite and was developed for use as a drying agent in steel and organic combustion analysis (Smith, Brown, and Ross (2)). It is comparable with phosphorus pentoxide in efficiency as a drying agent and has several other marked advantages. It does not become sticky while exposed to the air when charging the tubes. The spent portion differs in physical appearance from the unused and hence it is easy to determine when the tubes require recharging. The material has considerable absorbing capacity, and may be used until its weight has been increased by 20 or 25 per cent. The used material which is the hexahydrate form may be restored to the trihydrate form by heating *in vacuo* for 24 hours at 140°C . In use the hydration is accompanied by a contraction in volume which prevents caking and makes the tubes easier to clean. Each of the Blount tubes holds about 60 gm. of the magnesium perchlorate trihydrate in the two sides. This amount will absorb from 10 to 15 gm. of water before allowing any to pass on. The amount of water absorbed in the metabolism apparatus in which these tests were made was about 0.4 gm. per hour (with rats as experimental animals). Each metabolism test lasted from $\frac{1}{2}$ to 1 hour, hence from 25 to 60 determinations could be made with one charging of the tube. The amount of water given off from the CO_2 absorbent is much less than 0.1 gm. per hour, hence upwards of 100 to 150 determinations could be made with one charging of these tubes.

The carbon dioxide absorbent that has given the best results is the asbestos-sodium hydroxide mixture. This material is marketed under the trade name ascarite and was developed for use in determining the carbon in steels as CO_2 in combustion analyses (Stetser and Norton (3)). It has a number of distinct advantages over the other absorbents. It does not become sticky while charging the tubes. It is more efficient than soda-lime; smaller amounts may be used and the necessity of replacement is not so frequent. The granules are of the right size for use in the Blount tubes. The material is a very good drying agent itself and allows little of the water that is formed in the reaction of CO_2 and NaOH to pass on to the drying side of the tube. The material does not cake in the tube as much as does the sodium hydroxide and hence cleaning and recharging the tubes are easier. Another very decided advantage is the change in color from gray-black to white as it is used. It is possible to see at a glance when a tube needs recharging. The absorbent is very efficient and at the rate of flow of air through a metabolism apparatus absorption is complete in the first tube. Even when the first tube gains 0.5 gm. in weight the second rarely gains more than a mg. This asbestos-sodium hydroxide material absorbs from 20 to 25 per cent of its weight of CO_2 , and while this is not as much as shell NaOH takes up, the NaOH very often cakes to such an extent that it must be changed before it is entirely spent. One side of a Blount tube will hold about 40 gm. of the material which absorbs about 10 gm. of CO_2 . A medium sized rat produces on the average about 0.2 gm. of CO_2 per hour, and hence one charging of a tube will last for 40 to 100 determinations.

SUMMARY.

The use of two new absorbents for water and carbon dioxide in gravimetric metabolism determinations is described. Magnesium perchlorate trihydrate (dehydrite) as a water absorbent is free from the disadvantages attendant upon the use of sulfuric acid or phosphorus pentoxide. It is as efficient in absorption as phosphorus pentoxide and is believed to be nearly ideal as a drying agent in this type of work. The spent material may easily be restored to the trihydrate form by heating *in vacuo*.

A preparation of asbestos impregnated with sodium hydroxide (ascarite) has been found to be excellent for carbon dioxide absorption, with distinct advantages over the absorbents commonly used.

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THE NITROGENOUS GROUPS OF NUCLEIC ACID.

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The frequently discussed question whether or not the molecule of yeast nucleic acid contains a uracil group was almost decided by the work of Jones and Riley.¹ From a study of the rate at which phosphoric acid is set free from nucleic acid by acid hydrolysis they concluded that the number of pyrimidine groups in nucleic acid is equal to the number of purine groups, that is to say, there are two of each. This conclusion however leaves open one remote possibility; namely, that the molecule of yeast nucleic acid in fact contains two pyrimidine groups but both may be cytosine groups.²

This somewhat improbable view of the case was apparently supported by the subsequent work of Jones and Perkins.³ From the products of hydrolysis of yeast nucleic acid by a very mild method with dilute sodium hydroxide at the room temperature, they were unable to isolate uracil nucleotide, but after numerous efforts we have been unable to show that twice as much cytosine nucleotide is produced in this reaction as one would expect. By a study of the rate at which carbohydrate is set free from nucleic acid Hoffman⁴ supported the idea set forth by Jones and Riley that there

¹ Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii.

² In dealing with this subject we are careful to take into consideration all theoretical possibilities because one finds in this field frequent and curious anomalies. For instance, nucleic acid can be decomposed into its nucleotides by two processes: first, by the action of the thermostable agent of the pancreas; second, by the action of dilute sodium hydroxide at the room temperature. In both cases nucleotides are exclusively formed, but in the former reaction the acidity of the material is not perceptibly changed while in the latter there is an unmistakable change. Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1923, lv, 557, 567.

³ Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924-25, lxii, 557.

⁴ Hoffman, W. S., *J. Biol. Chem.*, 1927, lxxvii, 15

are two pyrimidine groups in the molecule of this substance. While this discovery was only a verification from a different point of view of the previous findings of Jones and Riley, it was an undoubted instance which served to convince us of our previous contention that the nucleic acid molecule contains two pyrimidine groups, and since the work of Jones and Perkins had been executed under conditions that scarcely permit deaminization, we concluded that they must have lost material in their analytical operations. By an examination of the solutions from which Jones and Perkins precipitated the lead salts of the nucleotides we found a considerable amount of soluble nucleotide material, and if it should happen that the lead salt of uracil nucleotide has a considerably greater solubility than the lead salts of the other three nucleotides the failure to find uracil nucleotide would be explained.

Proceeding upon these lines we have found that the lead salt of uracil nucleotide has a specifically great solubility in dilute solutions which contain sodium acetate. This finding shows that the failure of Jones and Perkins and of Calvery⁵ to isolate uracil nucleotide from the hydrolytic products of yeast nucleic acid was due to a loss of material. In order to avoid such a loss it is necessary to decompose nucleic acid with a hydrolytic agent that can be removed before the lead procedure is undertaken. The following method meets this requirement and yields accurate results.

By a sufficiently prolonged hydrolysis with *ammonia at the room temperature* nucleic acid is split into its nucleotides. Neither purine bases nor phosphoric acid are set free and deaminization is not likely to occur under these conditions. The excess of ammonia can be removed before the lead salts of the nucleotides are precipitated so that their isolation will not be vitiated as in previous experiments⁶ by the solubility of the lead salt of uracil nucleotide in an acetate solution. After hydrolysis of yeast nucleic acid under these conditions we were able to isolate all four nucleotides and have found an excellent method for the decomposition of nu-

⁵ Calvery, H. O., *J. Biol. Chem.*, 1927, **lxxii**, 27.

⁶ The excess of sodium hydroxide was formerly neutralized with acetic acid thus giving rise to a solution which contained sodium acetate and therefore held in solution the lead salt of uracil nucleotide. This difficulty occurred not only in our own work but also in that of all others who have worked on this subject.

cleic acid into its nucleotides and subsequent quantitative recovery of the products. From 50 gm. of crude moist yeast nucleic acid after the frequent removal of small portions for tests, we obtained 48 gm. of crude nucleotides from which 44 gm. were obtained after the execution of the lead process.

EXPERIMENTAL.

50 gm. of yeast nucleic acid were dissolved in 1250 cc. of 20 per cent ammonia and the transparent red fluid was allowed to stand for 2 weeks at the room temperature. Occasional tests from time to time of small quantities removed for the purpose showed that the decomposition of nucleic acid is not complete in 6 days but is complete in 7 days. The nucleotide solution was filtered and exposed at the room temperature until free from ammonia and dry. The material was dissolved in 200 cc. of warm water and the faintly acid solution after neutralization with ammonia was treated with absolute alcohol for the separation of the ammonium salts of the nucleotides into the guanine fraction and the adenine fraction frequently referred to in our former papers. The analytical procedure from this point does not differ materially from that which has already been described many times by ourselves and others. All four nucleotides were obtained in crystalline form. Their analyses are given below.⁷

Guanine Nucleotide.

Micro-Dumas-Pregl for nitrogen

2 474 mg. gave 0 379 cc N at 23° and 754 mm.

Required for guanine nucleotide N 17 54.

Found " 17 52.

Adenine Nucleotide.

2 986 mg. gave 0 501 cc. N at 24° and 755 mm.

Required for adenine nucleotide N 19 18.

Found.. . . . " 19 16.

⁷ While we believe such to be true, nevertheless this article does not purport to prove that the molecule of yeast nucleic acid contains a uracil group. It shows that there is no experimental evidence to the contrary.

Cytosine Nucleotide.

4.386 mg. gave 0.498 cc. N at 22° and 756 mm

Required for cytosine nucleotide.

.. N 13 00.

Found.

. " 13 07.

Uracil Nucleotide

4.109 mg gave 0.315 cc N at 27° and 756 mm.

Required for uracil nucleotide

. . . N 8 64

Found.

. . . " 8 67.

Brucine Salt of Uracil Nucleotide.

6.583 mg. gave 0.392 cc. N at 24° and 755 mm.

Required for uracil nucleotide

..... N 6 79

Found.. . . .

. " 6 78.

A NOTE ON THE ENZYME URICASE.

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As early as 1860 it was shown by Stockvis (1) that extracts of animal organs had the power of destroying uric acid. His findings have been confirmed by a number of workers (2) and in 1907 Wiechowski (3) showed that the principal end-product of this enzymatic change was allantoin. He was able to show quantitative conversion of uric acid to allantoin by an extract of dog liver. Ackroyd (4) confirmed these results by perfusion experiments using the liver of a rabbit. Ascoli and Izar (5) in 1908 showed that after the uric acid was destroyed it could be resynthesized by the tissues under anaerobic conditions. Later (6) these same workers showed that the enzyme was capable of synthesizing uric acid from dialuric acid and urea but not from a large number of other substances which they tried, among them allantoin. Spiers (7) in 1915 repeated the work of the Italian investigators and was unable to confirm their findings in any particular.

In view of the significance of the findings of Ascoli and Izar, if confirmed, and in view of the fact that Spier's work has not been corroborated it seemed advisable to reinvestigate this whole problem. This is especially true since Rose (8) has pointed out that positive results of this nature are much more significant than negative results and that the question should be reinvestigated.

It may be advisable to state that Spiers did not exactly repeat the work of the Italian investigators. He used ox and dog livers instead of calf liver; he used a different method of determination of uric acid and he used lithium carbonate instead of sodium hydroxide. None of these variations should account for the differences in results.

In this study the directions of Ascoli and Izar have been followed

as nearly as possible in every particular except in the method of determination of uric acid. The colorimetric method of Benedict and Franke (9) was used. A large number of experiments has been carried out, of which only a few are reported here and these only in briefest form.

EXPERIMENTAL.

Standard Enzyme Solution—Fresh calf liver was obtained from the slaughter-house. While still warm it was ground to a pulp and suspended in 0.85 per cent warm salt solution (250 gm. of pulp per liter of solution). The mixture was kept in an incubator at 37° for 1 hour and subsequently shaken for an hour. It was then strained through muslin and the liquid preserved with 1 per cent chloroform and 1 per cent toluene. This was used as the standard enzyme solution.

Standard Uric Acid-Enzyme Solution.—This solution was prepared by adding to the standard enzyme solution above enough uric acid dissolved in warm N/10 NaOH to make approximately 0.4 mg of uric acid per cc. of solution. The general procedure for determining the uric acid content of this and subsequent solutions was as follows: The solution was freed from protein by adding 1 gm. of sodium acetate and 1 cc. glacial acetic acid per 100 cc. of solution and heating to boiling. When filtered a clear filtrate was always obtained which gave constant results when the uric acid was determined by the method of Benedict and Franke.

Experiment I—300 cc. of the uric acid-enzyme solution were placed in an incubator at 37° and a rapid stream of air was drawn through the solution for 72 hours. Chloroform, toluene, and water were added at various intervals to keep the volume nearly constant. At the end of this time the solution was diluted to the original volume and the uric acid content determined.

Uric acid before aeration	=	0.4	mg	per	cc
“ “ after	=	0.22	“	“	“

The solution was then aerated with CO₂ for 12 hours. The flask was stoppered and kept in an incubator at 37° for 72 hours. The uric acid was again determined. The solution contained 0.2 mg. per cc., showing a slight loss rather than any increase in the uric acid content.

Experiment II.—300 cc. of the standard uric acid-enzyme solution were aerated as before, made up to the original volume, and the uric acid content determined. It was then stoppered tightly and allowed to stand 3 days.

Uric acid content before aeration	=	0 41 mg	per cc
“ “ “ after “	=	0 15 “ “ “	
“ “ “ “ stoppering	=	0 15 “ “ “	

Experiment III.—This experiment was the same as Experiment I except H_2 was used instead of CO_2 after aeration.

Uric acid content before aeration	=	0 4 mg	per cc.
“ “ “ after “	=	0 09 “ “ “	
“ “ “ “ saturation with H_2	=	0 04 “ “ “	

Experiment IV.—In this experiment N_2 was used instead of CO_2 as in Experiment I and with very similar results.

Experiment V.—In this experiment H_2 was bubbled through the uric acid-enzyme solution rapidly for 3 hours. The flask was then stoppered tightly and let stand in an incubator for 3 weeks. At the end of this time the uric acid was almost completely oxidized. The oxidation was undoubtedly carried out by the oxygen absorbed in the solution which had not been replaced by the H_2 .

Experiment VI.—Carbon dioxide instead of H_2 was used as in Experiment V. At the end of a week there had been slight oxidation of the uric acid but when the solution was aerated with air for 3 days there was no change in the uric acid content, showing that the enzyme had undoubtedly been destroyed in the high concentration of CO_2 .

Experiment VII.—A blank on the enzyme solution showed that it contained 0 08 mg. of uric acid per cc. To 100 cc. of this enzyme solution was added 0.3 gm. of allantoin. The solution was stoppered tightly and incubated for 3 days. At the end of this time the uric acid content of the solution was too low to be determined. There had been no synthesis of uric acid from allantoin.

Experiment VIII.—To 200 cc. of the enzyme solution, containing 0.08 mg. of uric acid per cc. of solution were added 1 gm. of dialuric acid and 1 gm. of urea. The solution was stoppered and incubated for 3 days and the uric acid determined. It was too low to be determined accurately. Instead of an increase

in the uric acid there was a decided decrease. Uric acid was not synthesized from dialuric acid and urea under the conditions of this experiment.

SUMMARY.

1. The work of Ascoli and Izar on the resynthesis of uric acid from its decomposition products by the uricase of the liver has not been confirmed.

2. The results of Spiers have been corroborated.

3. Uric acid is oxidized by uricase under precisely the conditions which Ascoli and Izar claimed were necessary for the reverse of this process.

4. High concentrations of CO_2 over a period of several days destroy uricase.

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WHAT CONSTITUTES AN ADEQUATE SERIES OF PHYSIOLOGICAL OBSERVATIONS?

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(Received for publication, February 3, 1927.)

In a recent article which appeared in one of our well known journals the significant data were included in seven curves presented in a single chart. In this chart the horizontal axis measured time and the vertical one a physiological variable. We are informed that about 400 observations were used in the construction of the chart; that six animals were used in the construction of one of the curves and three in that of another. Of the distribution of the remaining observations we are told nothing. We are further informed that the points were located by the averages of similar observations. Presumably the average used was the arithmetical mean.

On the basis of this chart and in spite of the fact that we are trusted with none of the original data nor with any measure of deviation we are asked to believe a very interesting, and if true, important, conclusion. Since there is no possible means of judging the significance of the data on their own merits we are driven to the literature, there to find if possible, a series of individual determinations of the same physiological variable. Presuming these observations to have been obtained under comparable conditions it is possible to calculate a measure of deviation to take the place of the one that our author failed to include. After considerable search a suitable series of 86 observations was found. The mean deviation of this series was 15. The curve based upon six animals was the control curve and the mean deviation of the mean for this number of observations would accordingly be 6.1. The extreme range of the physiological variable as shown by the plotted points was found to be 17 units—less than 3 times the mean deviation of the mean of the control series. Moreover twice

the mean deviation of the mean for the control series covered every point on the chart except two, these two being in the curve that was based upon three observations. It is common statistical practice to assign significance to a difference between means only when this difference exceeds one and one-half times the sum of the mean deviations of the respective means. Any interpretable significance, then, at once disappears from all of the curves except one. Again referring to the series of 86 we find that the mean deviation of the mean for 3 observations, the number in this curve, is 8.5. Twice this value covers every point in the chart, so that this curve cannot be considered to differ from any of the other curves shown. Thus the chart loses all *interpretable* significance and in spite of the author's 400 observations, the interesting and important conclusion falls in the great limbo of the unproven. This is quite different from saying that it is untrue, but of its truth the reader has no means of judging.

Such a condition is peculiarly disappointing, for the author must have collected most of the data necessary to demonstrate the significance of his work in the course of the very research which he was reporting, and the remainder was undoubtedly available in the archives of the laboratory in which he was working. All of this information could have been presented in a chart without undue confusion and without the use of additional space in the text beyond that necessary to explain the symbols used, possibly six lines.

This is not an unusual example, though it is perhaps more striking than some, because of the large number of observations involved. Illustrations which are just as disappointing may be found in almost any volume of our journals, while papers which present so few observations that any analysis is impossible are so common as to be found in almost any number, several sometimes occurring in the same number.

The preceding paragraphs are not intended as a specific criticism of the paper which has just been subjected to analysis but only to illustrate more forcibly the fact that it is the first duty of an author to *demonstrate* that, within a reasonable degree of probability, his data have the significance that he attributes to them. In passing it may be noted that mere numbers do not necessarily do this.

The remainder of the paper will be devoted to the development of a method for judging of the value of data and of the number of observations which are necessary to give the data an interpretable significance.

In drawing conclusions from his work an investigator essentially makes the prediction that similar results will obtain if his work be repeated. The value of the prediction will, then, be measured by the probability of the predicted event occurring. The probability in turn is measured by any one of the measures of deviation, as the mean deviation or the probable error. Precision and variability both refer to the same phenomenon so that measures of one become measures of the other. Precision is also sometimes defined as the repeatability of an observation, and when so defined its relation to probability is obvious. Thus we are confronted at the outset with the theory of probability and the principles which underlie it.

One of the fundamental tenets of the scientific method is that prediction would be justified from a single observation if the material and conditions were perfectly controlled and if the observations were perfectly made and recorded. However, even in the sciences in which the material and significant conditions are comparatively simple, this is a practical impossibility. In order to meet the difficulty which thus arises it has become customary to make a number of observations under as nearly similar conditions as possible, with the hope that chance variations will cancel one another. By chance variations are meant those variations which arise from causes which are beyond our knowledge or means of control. The general conclusions (predictions) are based, then, upon the behavior of the *series* rather than on the response of any *single individual*.

Since resort is had to multiple observations only because the significance of a single observation is doubtful it becomes of importance to determine the number of observations which is necessary to constitute a significant series.

This question as well as that of the significance of such series was investigated in the present study by experimental treatment of 1000 consecutive blood sugar determinations (Table I). In addition to this series of 1000 determinations, illustrative use was made of a similar but independent series of 500 determinations,

TABLE I

The raw data of the series of 1000, presented in the order in which the observations were made

114	144	96	140	128	122	112	148	114	114	114	122	138	152	121	123	118	112	125	118
128	122	128	145	148	125	108	145	116	94	105	160	112	136	121	136	123	119	150	132
128	119	128	102	132	128	114	112	114	112	119	125	128	121	118	102	136	112	122	168
124	112	130	122	128	102	132	122	105	130	135	125	132	118	126	105	131	144	114	168
124	116	122	92	142	108	130	125	94	135	105	116	114	149	116	131	118	128	130	132
116	112	119	128	151	108	108	130	114	148	149	128	125	147	129	123	113	130	142	135
122	110	112	142	116	125	112	116	105	80	145	128	148	144	126	134	110	128	138	138
124	128	130	116	122	119	112	166	130	102	125	112	112	121	116	116	113	122	112	148
112	122	102	116	145	94	122	128	157	94	125	122	119	110	123	123	118	118	118	148
114	141	112	138	145	116	128	128	142	130	145	138	122	131	121	121	118	114	128	143
118	128	102	119	112	118	142	154	135	110	135	122	110	134	118	105	134	154	116	145
135	125	112	105	119	132	116	160	145	135	135	138	135	129	131	126	118	122	114	160
130	112	130	128	116	132	110	135	140	122	130	130	142	123	105	118	118	119	112	151
128	130	114	108	119	105	122	160	125	138	135	116	128	107	118	116	123	154	114	135
116	140	119	112	112	114	125	122	119	170	145	132	130	123	134	121	129	108	118	140
124	114	122	128	119	102	105	108	119	128	128	122	122	113	105	123	144	119	130	145
124	122	122	119	105	140	132	132	130	125	122	125	128	141	126	97	195	119	132	102
124	116	128	112	122	130	116	148	125	116	125	119	116	123	105	118	121	122	128	113
122	142	108	92	128	135	116	132	151	105	114	128	136	134	116	110	131	148	130	102
110	112	105	116	108	138	122	138	125	112	128	132	118	126	121	107	116	112	119	110
122	125	112	92	116	116	122	128	128	102	119	128	110	105	99	129	116	122	122	114
124	112	114	108	119	112	114	131	132	119	105	132	105	113	94	110	100	138	119	116
116	114	116	108	128	122	140	128	130	112	125	163	131	113	105	136	129	132	114	108
130	130	108	108	130	140	116	122	112	122	145	132	126	136	105	123	110	122	138	138
108	142	116	135	116	116	112	142	122	128	128	135	131	107	116	134	118	142	128	108
114	128	130	116	128	138	116	132	130	130	130	135	129	113	118	121	113	128	134	119
104	119	114	116	116	148	125	160	128	119	132	119	131	118	126	118	129	140	123	104
128	122	128	128	112	130	128	140	116	110	132	138	110	118	105	121	121	138	138	110
138	130	108	132	166	138	166	138	135	99	142	145	121	118	110	121	129	138	128	110
114	125	112	142	112	128	135	128	135	112	132	128	123	113	116	121	131	142	130	108
108	128	116	116	122	154	128	125	125	99	135	122	129	113	116	129	141	114	116	106
122	125	128	116	120	138	142	110	96	102	140	125	134	123	110	105	121	110	102	112
108	116	116	140	116	128	168	132	125	114	130	140	144	129	113	107	167	124	112	94
105	128	112	138	125	168	122	119	128	102	125	142	139	111	136	113	118	128	114	109
112	116	105	132	102	166	138	138	166	110	154	122	134	130	116	110	139	134	112	118

TABLE I—*Concluded.*

125	122	84	96	122	166	138	160	154	140	142	132	97	105	123	105	118	112	102	112
108	114	128	138	116	130	119	154	154	130	140	138	129	133	126	116	123	128	112	110
116	110	124	128	140	116	128	130	130	138	135	122	136	108	121	110	121	148	116	132
142	108	114	122	122	151	116	122	119	114	122	116	144	116	131	105	141	114	112	128
122	110	117	134	140	160	160	112	130	99	128	128	129	125	118	121	123	130	116	119
130	112	110	119	102	170	119	110	122	105	140	128	126	120	113	129	116	112	116	116
119	114	132	142	112	157	158	105	119	112	125	122	123	105	113	134	140	128	102	110
122	112	122	154	116	116	138	105	110	88	130	142	129	111	121	129	128	130	122	128
125	119	116	130	80	112	154	116	138	114	142	125	139	134	129	113	138	114	116	124
116	116	128	112	74	151	119	145	128	99	128	122	102	128	118	110	132	119	122	116
112	112	102	112	128	112	148	151	92	119	142	128	107	105	131	102	130	128	138	120
116	130	116	122	135	128	114	140	108	125	135	119	107	128	126	118	142	125	120	116
96	130	96	125	112	114	142	125	110	128	145	125	129	123	121	134	114	128	132	112
116	128	114	132	138	116	132	135	128	138	140	138	134	121	123	123	122	135	141	116
150	105	128	116	122	125	160	151	96	128	119	108	157	126	129	134	130	125	140	112

consisting of four groups of 100 each from our own laboratory and one group of 100 taken from the published work of another institution. These observations were made as the initial or control observation in blood sugar curve studies and so constitute a peculiarly appropriate series for a study of this type. They are consecutive in the sense that they are used in the order in which they were obtained, without selection on any other basis. The observations from outside sources are used in the order in which they appeared in the literature. There were no known variations in the nature of the animals, their care, or in the technique of analysis which would lead one to expect variations in the results. The variability of this material is of the same order as that of similar material as reported from other laboratories.

Our own series of 1000, measure of deviation

14 2

Clough, Allen, and Root (1923)

16 9*

Eadie (1923) . . .

12 2*

* Recalculated.

A discussion of the physiological significance of our own results in their proper setting is in preparation. Illustrative use will also be made of 100 heart rates and 100 blood pressures, these observa-

tions having been made on dogs under ether. They are taken from the publications of still another laboratory and are used in the order in which they appeared.

It must be borne constantly in mind that any prediction from such a series is founded on results which were obtained from material that was not strictly uniform and by a technique that was not perfectly controlled or perfectly described. From this it follows that no prediction can be absolute. A prediction, however, will have a certain probability which will be higher as the control of the material and conditions is perfected and as the number of observations is increased. The increased perfection of technique will be reflected in a decreased *amount* of variation and the increased number of observations will be reflected in a greater uniformity of variation when the variations of different series are compared. Both of these factors are of direct aid in increasing the probability of a prediction.

It is frequently desirable to represent the entire series by a single value. A value selected for this purpose is an average. There are several averages but the arithmetical mean is the one usually chosen, because under ordinary circumstances from the mathematical standpoint it represents the series better than any of the other averages (Chauvenet, 1868, p. 473). There are special cases, however, in which it should not be used (Mills, 1924, p. 111; Day, 1925, p. 160; Chaddock, 1925, p. 80).

When an average is used the question arises not only as to how well the series which we have obtained represents the general field, but also as to how well the average which has been selected represents the particular series. The faithfulness with which each reflects its particular field will be increased with the number of observations which have been made. Since physiological experimentation is frequently attended with a considerable expenditure of both time and money, it is of importance to know the minimum number of observations that will depict the field under investigation with sufficient precision to permit of safe prediction. For this purpose it is necessary to know three values:

1. The mean, which is a value selected to represent the particular series of observations under consideration.
2. The mean deviation (or some approximation to it) which is a measure of the individual variations within the series.

3. The mean deviation of the mean, which is a measure of the variability of the means of similar series within the same general field. The calculation of this value permits the prediction, within certain limits, of the true mean (the mean of an infinite number of observations) and of the distribution of the means of subsequent series about this true mean.

The method by which the arithmetical mean is calculated is already common knowledge and need not be further discussed. The mathematical significance of this mean is developed by Chauvenet (1868) and by Mellor (1909, p. 518).

The formula for the mean deviation (indicated in the present paper by ϵ) is:

$$\epsilon = \sqrt{\frac{\sum d^2}{N-1}}$$

where ϵ = the mean deviation.

d = the individual deviations from the mean; $\therefore d$, the difference between the individual observations and the mean of the series

N = the total number of observations in the series

Σ = a symbol indicating the "sum of."

There seems to be considerable confusion both in the nomenclature and usage of the several measures of variation. It is not the desire of the author to introduce any new terms or to criticize the practice of workers already in the field. To avoid confusion, however, it is necessary to note that statisticians make use of the standard deviation (σ), the formula for which is

$$\sigma = \sqrt{\frac{\sum d^2}{N}}$$

The derivation of the mean deviation is somewhat more rigid, though in truth both are approximations (Chauvenet, 1868, p. 493). Statisticians are justified in the use of the standard deviation since in general they are working with series in which N is very large and so not sensibly different from $N-1$. On the other hand in experimental work the minimum representative number is purposely used, so the difference between N and $N-1$ may often be significant. Since, then, at times it will be necessary to use the $N-1$ formula it would seem better, in the interest of uniformity, to make the practice general in physiological work.

When the deviations are very small or where the accuracy of the individual observations is very low the average deviation (A.D.) is sometimes used. The formula is.

$$\text{A.D.} = \frac{\sum d}{N}$$

Thus, it is used by physicists because of the very small deviations found in their work, and sometimes by economists because of the inaccuracy of their individual items (See, however, Kohlrausch, 1910) Chauvenet (1868, p. 493) uses the term mean error with the significance given to mean deviation in the present paper. The usage of Mellor (1909, p. 525) and of Kohlrausch (1910, p. 1) is similar. Some of the later writers especially among the statisticians apparently do not distinguish between mean deviation and average deviation (Mills, 1924, p. 149, Yule, 1922, p. 144). Since it is desirable to have a specific term for the measures of deviation derived by each of the formulae it would seem desirable to revert to the older significance of mean deviation and to use average deviation only when it is desired to indicate the term derived from the simpler formula. It is to be noted that this necessitates most careful distinction between mean and average deviation because of the colloquial significance of these two terms.¹

From the theory of probabilities, about two-thirds of all of the observations should not be farther from the mean than $\pm \epsilon$; about 95 per cent should be within $\pm 2\epsilon$; 99 per cent within $\pm 3\epsilon$; and observations more distant from the mean than $\pm 4\epsilon$ should be very rare indeed. The statement just made is dependent upon the character of the distribution and does not hold closely unless this is such as to give the typical bell-shaped curve. Given a symmetrical distribution and a sufficient number of observations to give stability to the mean and mean deviation, a prediction based on the above theory should hold approximately for all subsequent observations of a similar nature. In this manner a series may be

¹ The mathematical development of the theory of the formulae is somewhat involved and since it may be found in a number of excellent texts it is not included here. The titles of a few of these texts are given for the convenience of those interested in this phase of the problem. Chauvenet, W., *A treatise on the method of least squares*, Philadelphia, 1868. Merriman, M., *A textbook on the method of least squares*, New York, 4th edition, 1888. Bertrand, J., *Calcul des probabilités*, Paris, 1888. Mellor, J. W., *Higher mathematics for students of chemistry and physics*, London, 3rd edition, 1909. Wright, T. W., and Hayford, J. F., *The adjustment of observations*, New York, 2nd edition, 1906. Palmer, A. de F., *The theory of measurements*, New York, 1912. Weld, L. W., *The theory of errors and least squares*, New York, 1922. Fisher, A., *The mathematical theory of probabilities*, New York, 2nd edition, 1922. Fisher, A., *An elementary treatise on frequency curves*, New York, 1922.

The practical use of the formulae is well treated in the texts by Chaddock, Day, Mills, or Yule, referred to in the bibliography. There are numerous other good texts on this phase of the subject but the one by Kelley, T. L., *Statistical method*, New York, 1924, should be especially helpful.

tested to determine whether or not it contains a sufficient number of observations. This method will be developed more fully later (see Tables III to V).

The values of the individual observations will vary among themselves as will the amounts by which they differ from one another, while both of these variables will differ from series to series (see Figs. 1 to 4 and 7 to 9). It follows from this that the truthfulness with which given means depict their respective series will vary and that a measure of the significance of the mean becomes necessary. Theoretically the mean deviation of the mean is such a measure. This may be derived from the mean deviation (ϵ) by the following formula:

$$\epsilon_M = \frac{\epsilon}{\sqrt{N}}$$

or from the original data by:

$$\epsilon_M = \sqrt{\frac{\sum d^2}{(N-1)N}}$$

The significance of the symbols is the same as in the previous formulæ with the addition of ϵ_M , which represents the mean deviation of the mean. For the derivation see Chauvenet (1868, p. 490), Mellor (1909, p. 521). Chaddock (1925, p. 235) discusses the significance of the mean deviation of the mean but does not develop the formula.

Again referring to the theory of probabilities, we learn that the chances are two out of three that the mean of a given series does not lie farther away from the true mean (the mean of a series containing an infinite number of observations) than $\pm\epsilon_M$. They are 95 out of 100 that it is not more than $2\epsilon_M$ from the true mean, and 99 out of 100 that it is not more than $3\epsilon_M$ distant. This probably will hold strictly only when the distribution of the original observations is such as to give a symmetrical bell-shaped curve. Neglecting any effect of an asymmetrical distribution or assuming it to be symmetrical, it may be noted that such a distribution of the means implies that the means of similar series will arrange themselves about the true mean in the same manner that the individual observations are arranged about the mean of a given

series, save only that in the former case ϵ_M is used as the measure of probability, while in the latter ϵ is used (see Figs. 7, 9, and 10).

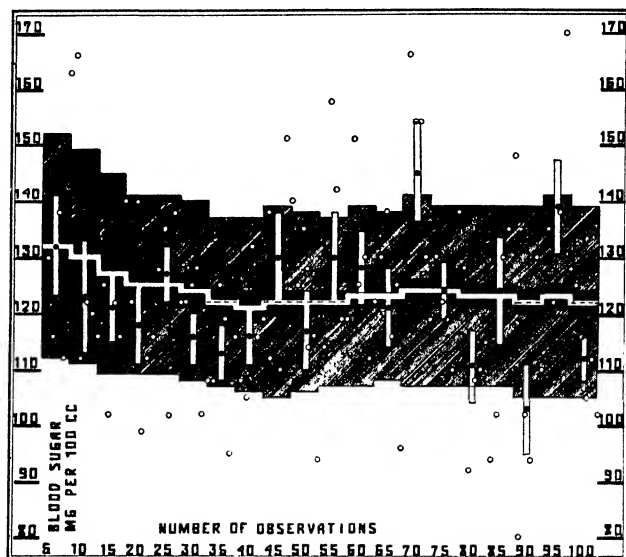


FIG 1 The growth and stabilization of a series of blood sugar observations. In the figure are shown (1) The individual determinations in the order in which they were obtained (circles), (2) the means of the successive series of 5 determinations each (dots), (3) the mean deviations for these successive series (vertical bars), (4) the mean of the cumulative series (heavy, horizontal white line), (5) the mean deviation of the cumulative series (diagonally hatched field), (6) the mean deviation of the mean of the cumulative series (solid black field); (7) the final mean of the cumulative series is carried back through the diagram by a fine dotted line. In the first instance the two systems are identical, since each consists of the first 5 observations of the grand series. In the second instance the two systems have separated and remain so throughout the remainder of the diagram. Note particularly the scatter of the observations; also note that the range is not covered until some 40 to 50 observations have been made, and that stabilization of the mean takes place at about the same time. The great deviations of the mean and of the mean deviations of the short series are obvious.

From what has been said it is evident that, before a series can have an interpretable significance it must contain a sufficient number of observations so that both its mean and its mean devia-

tion will have assumed a certain degree of stability. *The most important point to be determined, then, is the minimum number of observations which will permit of a safe generalization.* After this, it is of interest to determine the effect of additional observations upon the precision of the result. It may also be of value to consider the significance of generalizations which are based upon an

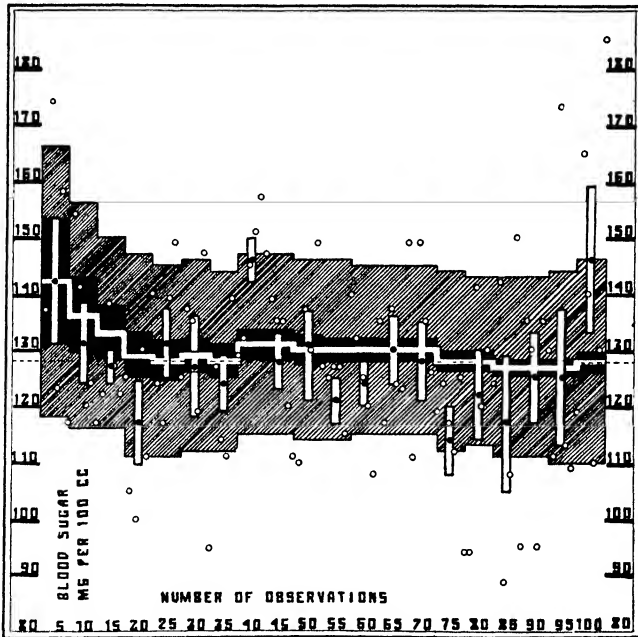


FIG 2 The material and significance of this figure are similar to those of Fig 1. The material was, however, derived from a different laboratory. Note that the apparent stabilization of the mean at 20 is dependent solely upon the arrangement of the next few observations and had almost any other combination shown on the chart occurred at this time, the stabilization would have been delayed.

insufficient number of observations. This same material also offers an opportunity for the study of the worth of the theory of probabilities as a means of describing an actual series and as a basis for making predictions from a completed series.

Since the physiological significance of the material under treatment is not the present concern, it will suffice to say that the

observations were obtained by the use of standard experimental methods, and that the deviations in our results are of an order common in careful work of a similar character. (p. 85.)

Before beginning a formal analysis of the series of 1000, it will be helpful to follow the development of a typical series, from the first observation to a point where the observations are sufficiently

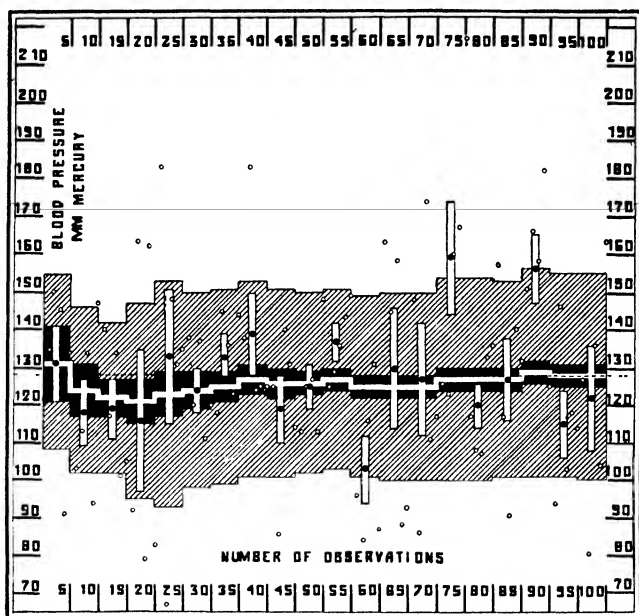


FIG 3 The material shown in this and the next figure was obtained from still a different laboratory. The measurements shown in this chart represent blood pressures taken by a mercury manometer on dogs which were under ether anesthesia. The significance of the figure is similar to that of Fig 1.

numerous to permit predictions which have a high degree of probability. Fig. 1 illustrates the stabilizing effect of added observations and the general behavior of a series while it is being built up. This figure shows (1) the individual observations in their chronological order (circles); (2) the means of the consecutive groups of 5 observations (dots); (3) the mean deviations of the means, calculated separately for each of these groups of 5 (ver-

tical bars); (4) the mean of the cumulative series, by *increments* of 5 (heavy, horizontal white line); (5) the mean deviation of this cumulative series, by increments of 5 (diagonal hatching); (6) the mean deviation of the mean of the cumulative series, calculated by increments of 5 (solid black); (7) the grand mean of the completed series—the final mean of the cumulative series—carried back through the diagram by a fine dotted line.

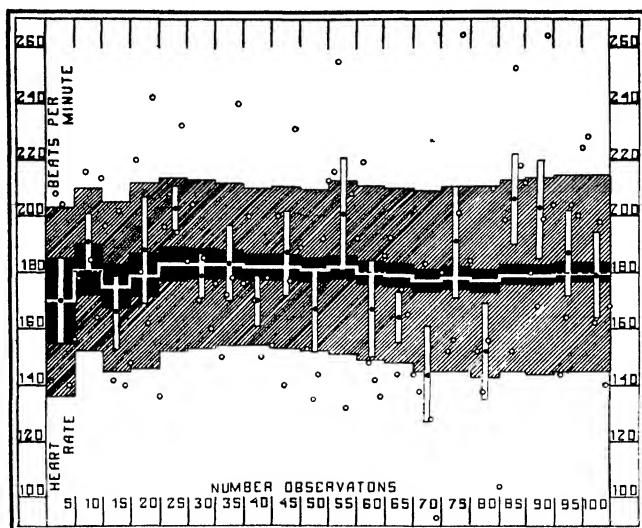


FIG 4. This chart depicts the development of a series of heart rates. The rates were taken on dogs under ether. The apparent early stabilization of the mean in this case, like that shown in Fig 2, has but little significance as will be seen by comparing the distribution of the early observations with that of those coming after the stabilization has taken place. A mean acquires a certain inertia and when it once approaches the probable position of the true mean it is difficult to dislodge it. Had the observations been plotted in the reverse order the picture would have been very different.

The range of the "scatter" of the individual observations will receive attention first, for it should be apparent that an adequate measure of scatter is not possible until the entire range is fairly covered by the individual observations, and that the series must be sufficiently long to enable this to be done. The approximate number necessary to cover the range may be estimated from the

diagram. The author's estimates are given in the legends below Figs. 1 to 4. The great variations in the level of the means and in the amount of variation, indicated by the variations of the mean deviations of the means, vertical bars, should make it obvious that

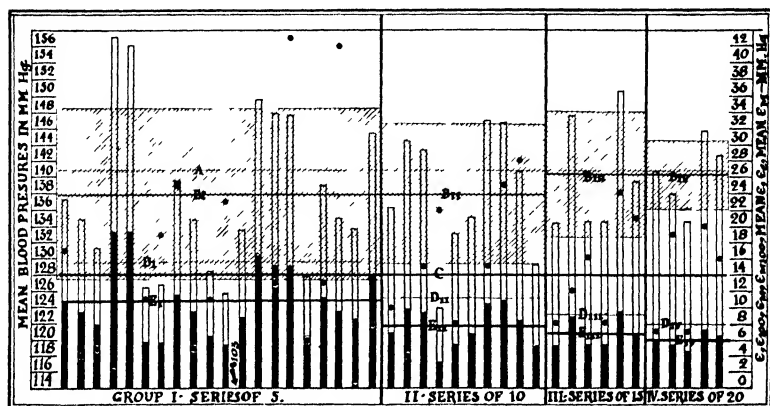


FIG 5 This figure gives a more complete analysis of the material shown in Fig 3 than was possible by the method used in that figure. It is designed to illustrate the fluctuations of M and ϵ with short series and their relative stabilization as the series increases in length. The material is not sufficient, however, to permit the study of a significant number of component series in which the stabilization had become satisfactory. The series of 100 observations was broken up into component series of 5, 10, 15, and 20 observations each. The means of these component series are indicated by dots, that of the 100 observations by the line C . The mean deviations of the component series are shown by the total height of the bars; that of the complete series by the line A . The variability of the mean deviations (ϵ) is measured by their mean deviation (ϵ_e) and is indicated by the diagonal hatching. The lines designated by B_I , B_{II} , B_{III} , and B_{IV} indicate the means of the mean deviations of the respective groups. The mean deviations of the means (ϵ_M) of the component series are indicated by the solid portions of the bars. The mean deviations of the means for series of the respective lengths, but calculated from the mean deviation of the complete series, are shown by the E lines, while the empirical mean deviations of the means are indicated by the D lines.

5 is not an adequate number of observations, though it is of an order which frequently occurs in current journal articles.

Turning the attention from the independent series of 5 to the mean of the cumulative series (heavy white line) it will be seen that

it seeks the grand mean of the series (fine dotted black line) varying on either side of it with an ever decreasing range, as the number of observations is increased. The mean deviation (ϵ) tends at the same time to become constant, while the mean deviation of the mean (solid black) continually decreases, since it is the mean deviation divided by the square root of the continually increasing number of observations. This behavior of ϵ and ϵ_M is also shown in Figs. 2 to 5 and in Figs. 8 and 9.

The principles exemplified in Fig. 1 are further illustrated in Figs. 2 to 4, in which the material was treated in the same manner. Fig. 2 represents experimental material similar to that treated in Fig. 1. This material was, however, taken from the literature and is not from our own laboratory. Figs. 3 and 4 depict, respectively, the behavior of the series of blood pressures and of heart rates mentioned on p. 85. These figures will all repay careful study.

In Fig. 5 the series of blood pressures shown in Fig. 3 is treated somewhat differently. Here the series of 100 observations was broken up into shorter or component series of 5, 10, 15, and 20 observations each. The means of these component series are indicated by dots, that of the 100 observations by the line C . The mean deviations of the component series are shown by the total height of the bars; that of the complete series by the line A . The variability of the mean deviation (ϵ) is measured by its mean deviation (ϵ_ϵ) which is indicated by the diagonal hatching. The lines designated by B_I , B_{II} , B_{III} , and B_{IV} indicate the means of the mean deviations of the respective groups. The mean deviations of the means (ϵ_M) of the component series are indicated by the solid portions of the bars. The mean deviations of the means for series of the respective lengths, but calculated from the mean deviation of the complete series, are shown by the E lines, while the empirical mean deviations of the means (see p. 103) are shown by the D lines.

Presented in this manner this series shows that even many more observations than 5 may be unsatisfactory. Thus we find that neither the level (from the mean) nor the probability (from the mean deviation) can be predicted with any satisfactory degree of precision from 5 or even 20 observations. The introduction of the mean deviation of the mean deviation in this figure provides

a measure of the variability of ϵ . As, postulated on p. 86 it will be observed that this variability becomes less as the series becomes longer.

Likewise the series of 500 blood sugar determinations was broken up into component series of 5, 10, 20, and 100 observations. Thus there were 100 series of 5 observations, 50 of 10, 25 of 20, and 5 of 100 observations each. The series studied in Figs. 1 and 2 were, respectively, the first and fourth series of 100 shown in *D* of Fig. 6. The means of the component series are shown in Fig. 6 (black dots) in their chronological order. The grand mean of the 500 is shown by the heavy horizontal line which runs through the figure. The vertical lines with the arrow heads indicate $\pm 4\epsilon_M$. Consecutive means which differ by an amount which is commonly considered to be significant are connected by thin lines. Referring to this figure it is interesting to speculate on the value of conclusions which might have been drawn from any one of a number of pairs of consecutive means which differ from one another by what ordinarily would be considered a perfectly safe margin. An investigator trusting to such differences might well be sorely embarrassed by subsequently refuting his own work. The means of the four series of 20 observations each, shown in group C of Fig. 6 and connected by fine lines, offer an especially apt illustration of this type of danger. Here the mean of the first of the four series differs from that of the second by more than $4\epsilon_M$, certainly a significant amount. The third agrees with the first and the fourth with the second. Thus assuming that the first and third means represent control series while the second and fourth represent series that have been exposed to some experimental conditions, it would have resulted that had the investigator said "abracadabra" while drawing the blood of the second and fourth samples a profound and hitherto unknown biological "law" would have been "discovered." The evidence for this new law would have been considerably better than that submitted in support of much that is actually and seriously presented. It will be noted that within this same section of Fig. 6 there is also a group of three means which well might cause trouble. Again in Fig. 5, part IV, there is an almost exact counterpart of the "abracadabra research."

The series of 1000 was treated in a similar manner and this series

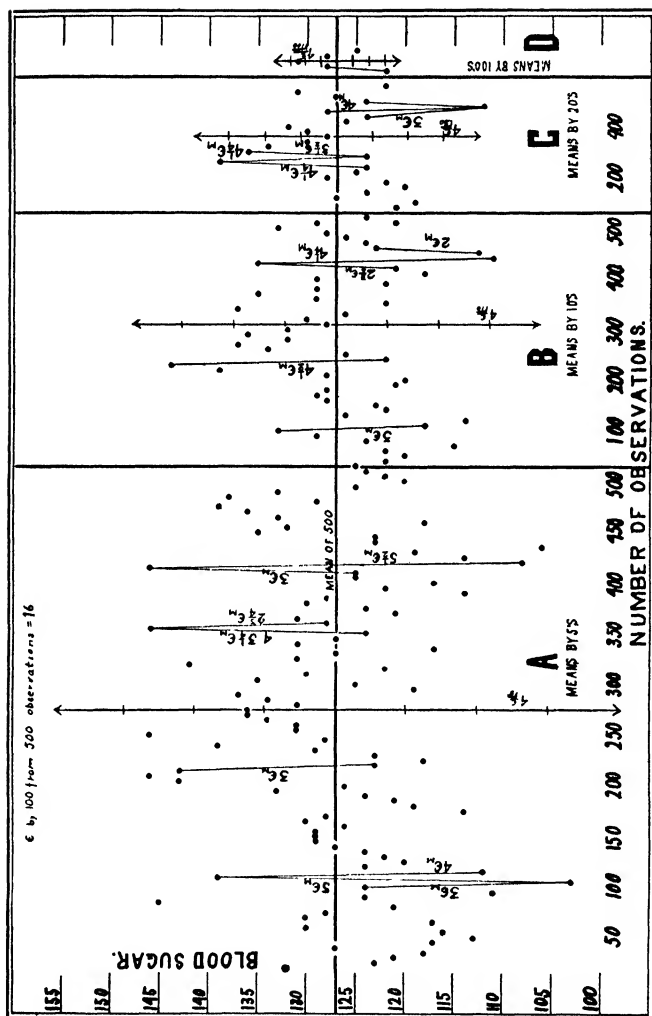


FIG 6. A series of 500 blood sugar determinations has been broken up into component series of 5, 10, 20, and 100 observations each. The means of the consecutive series are indicated, by the dots, in their natural order and in their relation to the grand mean of the 500 $\pm 4\sigma$ is indicated by the total lengths of the vertical arrows. The means of a few consecutive series which differ from one another by considerable amounts have been connected by light lines. The possibilities for drawing meaningless conclusions are numerous and obvious. In this regard note especially group C.

TABLE II

A study of the behavior of the mean deviation and of the mean deviation of the mean, based upon an analysis of the series of 1000, for the various values of N and for the several component series with given values of N . Column 1, the number of observations in each component series of the respective groups. Column 2, the number of component series in each group. Column 3, the mean of the means of the component series within each group. Column 4, the mean of the mean deviations of the component series within each group. Column 5, the empirical mean deviation of the mean deviations of the component series within each group (ϵ_e). Column 6A, ϵ_e calculated by the formula, $\frac{\epsilon}{\sqrt{2N}}$, where ϵ was derived from the first series of the respective groups, as given in Column 8 of Table III. While this value is not used in the discussion it is given for comparison with the value of ϵ_e given in Columns 5 and 6B of this table, since under ordinary circumstances it would represent all of the observations available. Column 6B, ϵ_e calculated by the same formula but from the mean value of ϵ for the group, Column 4 of this table. Column 7, the empirical mean deviation of the means (ϵ_M) calculated as described on p. 103. Column 8, the mean deviation of the mean calculated for series of the several lengths from ϵ_{1000} , by dividing it by the square root of N for the respective series. Column 9, the mean deviation of the mean, shown in Column 8, $+ 2.7$.

(1) No of observa- tions in the series N	(2) Series in the groups n	(3) Mean of the means $\frac{\Sigma M}{n}$	(4) Mean of the mean devia- tions $\frac{\Sigma \epsilon}{n}$	(5) Empiri- cal ϵ_e	(6) $\epsilon_e = \frac{\epsilon}{\sqrt{2N}}$		(7) Empiri- cal ϵ_M	(8) $\frac{\epsilon_{1000}}{\sqrt{N}}$	(9) $\frac{\epsilon_{1000}}{\sqrt{N}} + 2.7$
					A	B			
					ϵ from 1st series of the group	ϵ from Column 4			
5	200	124	11.2	5.1	1.8	3.6	8.9	6.3	9.0
10	100	124	12.1	3.8	1.3	2.7	7.5	4.5	7.2
20	50	124	12.7	3.3	1.1	2.0	6.8	3.2	5.9
30	33	124	13.0	3.3	1.0	1.7	5.8	2.6	5.3
40	25	124	13.2	3.0	1.0	1.4	4.9	2.2	4.9
50	20	124	13.3	2.8	1.2	1.3	4.6	2.0	4.7
100	10	124	13.2	2.8	0.7	0.9	4.1	1.4	4.1

because of the greater number of observations offers the opportunity of studying component series of greater lengths than was possible when using the material so far studied. The 1000 observations (Table I) were divided into series of 5, 10, 20, 30, 40,

50, and 100 observations each, and in each case all of the series of a given length were studied as a group. For example, with 5 observations in a series, there were 200 series in the group; with 10 observations in the series there were 100 series in the group; with 100 observations in the series there were 10 series in the group. The number of observations in the series is indicated by N ; the number of series in the group by n .

The means, mean deviations, and various other functions were determined and such of them as are of use in this treatment are collected in Table II, or are shown graphically in Figs. 7 to 10. Figs. 5 and 9 show, as has already been pointed out, a considerable variation among the mean deviations of any one group. This variability of the mean deviation is especially marked in the groups of shorter series. The same phenomenon is shown in a more general form in Column 5 of Table II and in B of Fig. 7, where the mean deviations of the mean deviations (ϵ_e) are presented. It is evident that the truthfulness of any prediction must depend directly upon the stability of the functions upon which it is based. In this case the function is the mean deviation (ϵ). It should be noted that the mean deviations of the mean deviations (ϵ_e) shown in Table II have been calculated directly. This was done by considering each ϵ in a given group as an original observation, and calculating ϵ_e by the formula for ϵ given on p. 87. The values so found for ϵ_e , and given in the table, therefore, represent the variations of the mean deviations of the respective groups which have actually occurred in the 1000 observations.

There is also a theoretical formula $\left(\epsilon_e = \frac{\epsilon}{\sqrt{2N}}\right)$ which permits the calculation of this same function directly from the data of a single series (Chaddock, 1925, p. 239). The various values calculated by the two different methods are given in Columns 5 and 6 of Table II. In Column 6A, ϵ_e is calculated from ϵ of the first series of the respective groups because ordinarily this would be the only material available. In Column 6B it is calculated from the mean value of ϵ for the group as given in Column 4 of the same table. The values in Column 6 were calculated by means of the above formula. A comparison of the corresponding values in these columns shows that the actual variability, as shown in Column 5, is considerably greater than would be expected from the theory, Column 6.

Practically this means that reliable predictions for work of this type cannot be made with the precision which the theory indicates.

In passing it may be noted that while the mean of the mean deviations (Table II, Column 3) is approximately constant through the different groups, it is not quite so. It becomes progressively larger as the length of the series increases. Apparently the reason for this is that the small number of observations contained in the shorter series does not ordinarily cover the range of distribution, so that there is usually a portion of the field towards one or both of its limits which is left unrepresented by any observations. This naturally shortens the range in all series in which it occurs and so results in an abnormally low mean deviation. *The mean deviations of short series are very apt to give the investigator a false sense of security which may well be misleading.* The longer the series the more adequately will the range be covered to its extreme limits and so the mean deviation must increase towards a maximum as the series more and more perfectly depicts the field. Since this maximum is approached as a limit it follows that the range of variability is limited, so ϵ_ϵ must approach a minimum as ϵ approaches its maximum, i. e. as ϵ approaches a constant. This minimum must theoretically be 0 but this ideal condition can never be attained in practical work. The number of observations which just render ϵ *sensibly* constant is the minimum number which will permit of a precise and at the same time a safe prediction. This behavior of ϵ is well illustrated in Figs. 1 to 4. In Fig. 5 the progressive approach of the means of the mean deviations for the several groups (the *B* lines) to the mean deviation of the complete series (the *A* line) with the increase in the number of observations, illustrates this increase of the mean deviation. The progressive decrease of the width of the hatched area likewise illustrates the decrease of the mean deviation of the mean deviations (ϵ_ϵ) which takes place at the same time. The behavior of the mean deviations of the mean deviations of the series of 1000 is shown in Fig. 7, part B.

Reference to Column 5, Table II, shows, as is to be expected from what has just been said, that ϵ_ϵ decreases as the number of observations increases. For a time this decrease is of sufficient magnitude to be obvious, but at 40 to 50 observations the rate of decrease becomes so slow that ϵ_ϵ becomes sensibly constant.

Thus ϵ_e for 100 observations is, as practically determined, the same as is ϵ_e for 50 observations. This would seem to indicate that 50 is the practical optimum number of observations for work

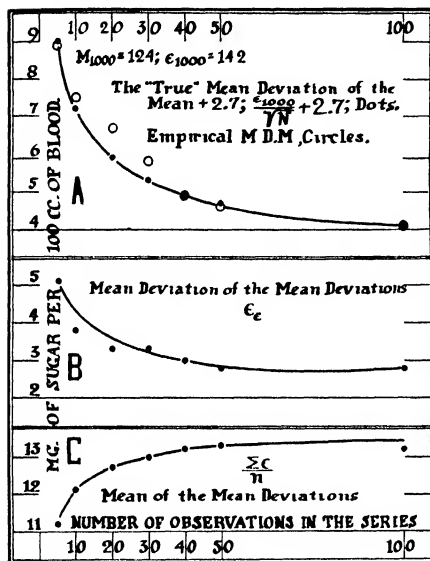


FIG 7

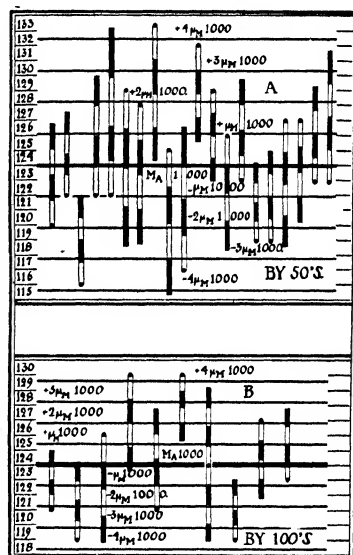


FIG 8

FIG 7 A graphic representation of some of the material which is presented in Table II. A, the position of the dots was obtained by the formula given in the figure. The empirical mean deviations of the means for series of the several lengths was calculated as described in the text. They are indicated by the circles. Note that the empirical ϵ_M is greater than the true values by a very considerable amount which is approximately constant. The meaning of parts B and C is discussed in the text.

FIG 8 A chart to show the relationship between the means of the component series of 50 and 100 and the mean of the series of 1000. This relationship is expressed in terms of ϵ_M . The mean of the series of 1000 is indicated by the heavy horizontal lines, ϵ_M for this series by the lighter horizontal lines. The means of the component series are indicated by the black dots and the mean deviations of their means by the bars which are extended towards the grand mean for a distance of $4\epsilon_M$ from the respective component means. Inadvertently μ is used in this figure for ϵ .

of this type; in fact there is no indication that 100 is any better than 50, though 50 is distinctly better than 40. If this is so it is necessary to confine our predictions to the limitations of prob-

ability of a series of this length (Fig. 7, part B). In Fig. 5 the progressive decrease in the width of the hatched area with the increase of the number of observations illustrates the same sort of thing for another type of observation.

Broken into constituent series in this manner, this same material offers another method of attack. Let it be assumed that the mean of the series of 1000 falls within twice its mean deviation from the

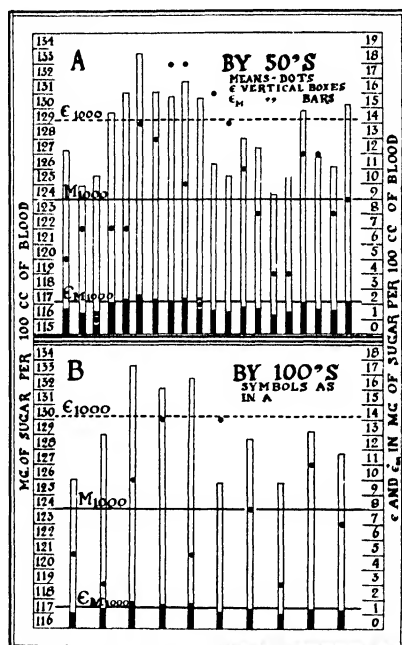


FIG 9 To show the relation of the mean deviations and means of the respective component series to the corresponding functions of the series of 1000 The variability of these values from series to series is well shown.

true mean. It follows then that it would not differ from the true mean by an amount which has any known physiological significance and that for practical purposes the two could be assumed to be identical. Likewise the mean deviations of the means calculated from the mean deviation of the long series by dividing it by the square root of the number of observations in the series of the respective groups could be assumed to be *practically* identical

with their true mean deviations. The empirical mean deviation of the means may be found by treating each mean of a given group as an original observation and calculating their mean deviation

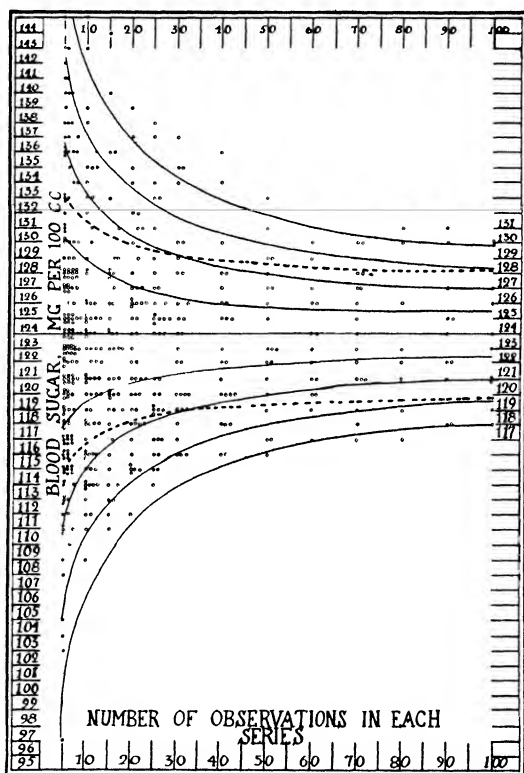


FIG 10 The distribution of the means of the component series, of the series of 1000, for various numbers of observations up to 100. The horizontal line through the center of the diagram is on the mean of the series of 1000. The solid parabolic lines above and below it represent, in order ± 1 , ± 2 , ± 3 , and ± 4 times ϵ_M . The dotted lines represent $\pm (\epsilon_M + 2.7)$. Note that approximately two-thirds of all of the means fall within the dotted lines as would be expected from the discussion in the text. The significance of this in making safe generalizations is obvious.

from the formula on p. 87. To illustrate: in the first group there are 200 means, each calculated from 5 observations. The arithmetical mean of these 200 means is 124 (M); this is subtracted,

algebraically, from each of the 200 (d). These differences are squared (d^2), added together (Σd^2), divided by 199 ($N-1$), and the square root of the quotient taken as the mean deviation of the means. This process was carried out for each of the seven groups and the results are collected in Table II, Column 7. The mean deviations of the means for series of similar lengths calculated from the mean deviation of the series of 1000 are given in Column 8 of the same table.

A comparison of the values in these two columns shows that in every case the empirical mean deviation of the mean is the larger by approximately 2.7. This is shown in Column 9 of the same table and in part A of Fig. 7. That is, the empirical formula for ϵ_M is $\frac{\epsilon}{\sqrt{N}} + a$, where a , in the present case, is 2.7, rather than 0 as would be expected from the mathematical theory. *Note particularly that this is not a generalization but simply a statement of the fact for this particular series* The practical significance of this deduction is that at least in the series here studied the mean is not as good a representative of the general field as the theory would indicate, and that when making predictions the discrepancy must be allowed for, either by the above formula or in some other manner. Conclusions or predictions based upon a close interpretation of the theory would surely be misleading. This is illustrated by the behavior of the several series represented in Figs 5 to 10. The theoretical meaning is not so clear but it could be interpreted as indicating some periodical variation in the material or technique, or possibly as resulting from the skew in the distribution.

Consideration of the variations of the mean deviations (ϵ_e) on p. 100 led to the conclusion that for the material studied, about 50 is the minimum adequate number of observations. The reasoning which has just preceded brings us again to the same conclusion, for it is with this number that the mean first assumes a practical stability. This is particularly well shown in Fig. 10. This same figure brings out the effect of the modification which it was found necessary to make in the formula for ϵ_M upon the possible precision of prediction. It will be seen that even $\pm 4\epsilon_M$ hardly covers all of the means and that there is a much larger portion of the means near the outer range than the theory would indicate. If, however,

TABLE III

The fulfilment of predictions concerning the 1000 observations made respectively from the first 5, 10, 20, 30, 40, 50, and 100 observations. The first series of the several groups was chosen as a basis for the predictions because, in the ordinary course of a research, prediction would naturally be made from all of the available observations, which would be the first, and only, series.

From the theory of probabilities 683 observations out of every 1000 would be expected to fall within $\pm\epsilon$ from the mean of a significant series. 955 should fall within 2ϵ from the mean and so on as indicated in the first line of the table. For the series of 5 observations only 391 were found to fall within these limits, $\pm\epsilon$ between 118 and 130 inclusively. In this particular case the failure of the prediction was due to the unusually close grouping of the observations in the short series for the mean is the same as that of the entire series of 1000. ϵ for the short series was, however, only 5.7 while that for the complete series was 14.2.

The general method for making the predictions will be clear from the example just given. In this table there is also shown the distribution of the 1000 observations as would be predicted from its own mean and mean deviation.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
No of observations in the series	No of observations within				Total No of observations	Means of the 1st series of each group	ε of the 1st series of each group	ε _M of the 1st series of each group
	± ε	± 2ε	± 3ε	± 4ε				
	Predicted							
	683	955	997	999+	1000			
Found								
N= 5	391	670	811	923	1000	124	5.7	2.5
10	324	665	818	917	1000	121	6.1	2.0
20	446	713	867	943	1000	121	6.7	1.5
30	482	780	957	957	1000	121	8.0	1.5
40	544	802	929	969	1000	120	9.0	1.3
50	690	924	969	997	1000	120	12.2	1.7
100	600	867	956	982	1000	121	9.8	1.0
1000	749	947	989	999	1000	124	14.2	0.45

the formula: $\epsilon_M = \frac{\epsilon}{\sqrt{N}} + a$, be used, where $a = 2.7$, it will be found that the distribution is about as would be expected.

Still another test of the value of a series is that of its actual use in the making of predictions, the truth of which may be deter-

TABLE IV

The predictions in this table were made in the same manner as were those in Table III, except that they were based consecutively upon each of the 20 series of 50 observations. In the next to the last line is given the mean fulfilment and in the last line the mean deviation of this fulfilment. It will be noted that this deviation is large for $\pm \epsilon$ and $\pm 2\epsilon$. Even at 3ϵ it is 2 per cent of the mean frequency and this frequency itself is 2 per cent below what it theoretically should be. The conclusion is forced upon us that these series do not justify predictions with a greater precision than 4ϵ .

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Order of series	No of observations within				Total No of observa- tions	Means of the several series	ϵ of the several series	ϵ_M of the several series
	$\pm \epsilon$	$\pm 2\epsilon$	$\pm 3\epsilon$	$\pm 4\epsilon$				
	Predicted							
	683	955	997	999+	1000			
Found								
1	690	924	969	997	1000	120	12.2	1.7
2	629	890	957	982	1000	122	9.9	1.4
3	506	814	937	971	1000	116	10.5	1.5
4	745	958	985	999	1000	122	14.7	2.1
5	782	967	999	999	1000	122	16.1	2.3
6	834	988	999	1000	1000	129	18.6	2.6
7	773	970	998	999	1000	128	16.1	2.3
8	607	950	995	1000	1000	133	15.8	2.2
9	827	970	998	999	1000	125	16.8	2.4
10	770	958	997	999	1000	117	16.7	2.4
11	499	878	964	995	1000	131	11.3	1.6
12	493	837	954	993	1000	129	10.5	1.5
13	657	935	982	999	1000	126	13.0	1.8
14	672	917	972	998	1000	123	12.3	1.7
15	556	802	933	969	1000	119	9.3	1.3
16	578	846	942	975	1000	119	10.4	1.5
17	776	954	999	999	1000	127	14.8	2.1
18	603	914	981	998	1000	127	11.8	1.7
19	646	911	967	992	1000	123	11.1	1.6
20	753	963	996	999	1000	124	15.2	2.2
Mean	669	918	976	993	1000	See Table II.		
Mean deviation	105	56	23	10				

mined. The fulfilment of predictions concerning the 1000 observations, made, respectively, from the first 5, 10, 20, 30, 40, 50, and 100 observations will be found in Table III. In this table will also be found the distribution of the 1000 observations so that it may be compared with the distribution that would be expected

TABLE V

This table is similar to Table IV, except the fulfilment of predictions concerning the 1000 observations which are based in turn upon each of the 10 series of 100. The data are presented as in Table IV. It will be noted that the predictions based upon 100 observations have but very little greater precision than those based upon 50. This is as would be expected from the discussion of Table II.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Order of series	No. of observations within				Total No. of observa- tions	Means of the several series	ε of the several series	ε _M of the several series
	± ε	± 2ε	± 3ε	± 4ε				
	Predicted							
	683	955	997	999+	1000			
Found								
1	600	867	956	982	1000	121	9.8	1.0
2	697	929	975	999	1000	119	13.0	1.3
3	834	980	999	1000	1000	126	17.6	1.8
4	712	963	996	999	1000	130	16.1	1.6
5	818	968	999	999	1000	121	16.8	1.7
6	519	872	965	995	1000	130	10.9	1.1
7	680	934	982	999	1000	124	12.7	1.3
8	578	846	947	975	1000	119	9.8	1.0
9	663	935	988	998	1000	127	13.2	1.3
10	672	917	972	998	1000	123	11.7	1.2
Mean	677	921	978	994	1000	See Table II		
Mean deviation	98	46	18	9				

from its mean and mean deviation. This method also indicates that prediction cannot be made safely from less than about 50 observations. In Table IV will be found the fulfilment of predictions based consecutively on each of the 20 series of 50 observations each. It was just learned from Table III that a series should

contain at least 50 observations. Table IV teaches that, even given a series of 50 observations, safe prediction cannot be made with a precision better than about 3 or 4 times the mean deviation. This again is in agreement with conclusions which were drawn from previous methods of attack.

The material presented in Table V is similar to that in Table IV except that the predictions are based on the consecutive series of 100 each. This table shows, as would be expected from consideration of Table II, that 100 observations are but very little better than are 50.

Occasional papers appearing in the literature reveal a certain naive faith, on the part of the authors, in the finality of the mean, regardless of the nature of the material which entered into its computation. Apparently, to these authors, almost any series of numbers may be added together and when the sum is divided by the number of items, the quotient acquires a peculiar significance. On the contrary, there are others who profess to question the propriety of attaching any importance at all to the mean of physiological data. Such authors prefer, rather, to pin their faith upon the individual observations. Though each of these attitudes betrays the lack of a proper comprehension of the purpose or significance of the mean, the latter is by far the better practice of the two. The treatment of data without the use of an average implies the ability on the part of the author and reader to keep each observation with its attendant circumstances of the research clearly in mind throughout the argument. With adequate series, this might, when the research is at all complicated, run well into the hundreds. With very short series the practice may, perhaps, be possible but from the outlook of the present discussion it apparently would not make much difference what method was used under such circumstances.

When observations become sufficiently numerous to give an adequate idea of the field from which they were drawn, the task of evaluating the series from the individual observations becomes quite impossible for most of us and some simplification becomes necessary. The ideal presentation would be that of a frequency curve for each of the series being considered. When such curves are shown on the same coordinates we have the best possible means of comparing the series which they represent. The prin-

cial difficulty lies in the fact that a really significant frequency curve requires many more than 50 observations, though by rather special treatment something may be learned from a series as short as 50.

As an alternative, one of the averages may be used; usually the arithmetical mean is chosen. An average is nothing more than a

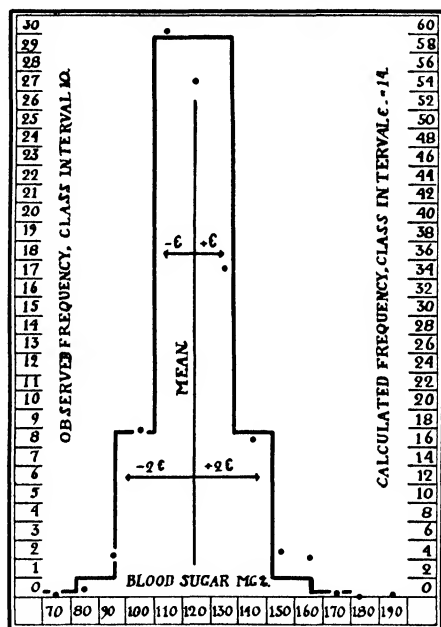


FIG 11 The solid line represents a frequency curve constructed directly from the mean and mean deviation of the series of 1000. The dots represent the actual distribution of appropriate classes. The figure illustrates the value of the mean, when taken in conjunction with the mean deviation as an aid in visualizing the behavior of a series of observations.

value selected to stand for the series. Taken alone none of the averages will give much information in regard to the series which it is selected to represent, and with the exception of the mode, almost none with regard to an individual observation. On the other hand an average taken together with a measure of deviation may have a very definite significance. With the mean and the mean deviation of an adequate series in hand a rough frequency

curve may readily be imagined, or even constructed (Fig. 11). A prediction may be made as to the mean value of subsequent series and the probability that this predicted value will fall within a specified distance from the true mean may be stated. The range of the distribution of the individual observations and their distribution over this range may be visualized. It must be distinctly understood, however, that the average of a series cannot be directly compared with any single observation (unless the average used be the mode or median). The average with its measure of deviation represents the series and *hence its general significance must be greater than that of any single observation*. This is in fact as it should be for it is the end towards which we have been working from the moment when we began to multiply our observations. This we did when we were convinced that a single observation had of itself but little significance. This fact that the average refers to the series rather than to the individual probably gives rise to a misconception upon which is based much of the criticism of its use. It was selected to represent a *series*, but when it fails to predict the behavior of an *individual* it is inconsistently discarded.

The very thesis of this paper would preclude the formulation of general conclusions upon the basis of the work here reported. The following running summary, however, may not be out of place.

Naturally the number of observations necessary will depend upon the requirements of the research and upon the variation of the individual results.

It was shown for the blood sugar of rabbits in the series of 1000 that approximately 50 observations were necessary to give a predictable precision and that with this number the precision attained is about 4%. As a matter of convenience this may be called the *significant precision* for this type of observation. This precision was not materially better for series containing 100 observations than it was for those containing only 50.

A more superficial study of 100 blood pressures of dogs under ether and of a series of heart rates under similar conditions indicated that for these measurements a like number is necessary and that approximately the same precision is attained.

From this it would seem that researches involving any of these

types of observation should present at least this number of observations to establish each point, and that conclusions requiring a precision better than 4ϵ should be avoided, unless and until it be *shown* that a lesser number is sufficient or that a greater precision is actually obtained.

The minimum number of observations in other fields necessary to establish a degree of precision and the degree of precision attained await investigation. Until this information is at hand it would seem incumbent upon an investigator to establish the adequacy of his data and the degree of precision as an integral part of his research.

The *determination* of the least adequate number of observations is not possible in the absence of a series far in excess of the actual number required. The number may be *approximated*, however, by several methods. One of these consists in building up the series by increments of some convenient number until the mean and mean deviation have become stabilized, as is done in Figs. 1 to 4.

The methods of attack and presentation which have been discussed are applicable to all forms of numerical data. Wherever the mean is used to represent a series it should be accompanied by the mean deviation (ϵ). Either, without the other, can have but little value in depicting a group of observations.

When the means of two or more groups are to be compared, as when observations are made before and after the imposition of experimental conditions, each mean should be accompanied by its mean deviation, since the precision of the mean is measured by its mean deviation (ϵ_M).

Any difference between two means cannot be considered as significant unless it at least approaches the sum of the significant precisions of the respective means. In the types of observations here studied this significant precision for series of from 50 to 100 observations was found to be about $4\epsilon_M$. Note that this is not a generalization but a specific fact for the series studied. In other fields and for longer series it may be more or less and must be specifically determined for each type of observation.

While, in the interests of uniformity, it is suggested that the mean deviation be used as the measure of precision in physiological work, the purpose of the paper is rather to emphasize the necessity of the recognition of the general problem. Any measure of

precision would be a great step in advance of the guesswork now so frequently encountered.

The author acknowledges his indebtedness to many of his colleagues for advice and information, especially to Professors R. E. Chaddock and H. B. Williams and Mr. H. F. Pierce for help with the theory or in the preparation of the manuscript, and to Mr. Louis Dotti for his interest or care in conducting the blood sugar determinations which formed the basis of the analysis.

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QUANTITATIVE DIFFERENTIATION OF VITAMINS A AND D. I.*

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The finding of Steenbock and Nelson,¹ confirmed under somewhat different experimental conditions by Drummond, Coward, and Handy,² that the weight curves of rats whose growth is inhibited by shortage of fat-soluble vitamin may respond to irradiation either of the animals or their food has led us to investigate further the method developed in this laboratory for the quantitative determination of vitamin A.³ This method, based upon the earlier work of Drummond, Coward, and their associates, now involves the use of experimental animals (rats) of accurately known age and nutritional history. The rats are placed when 4 weeks old upon a basal diet excellently adapted to the needs of the animals in all other respects but devoid of vitamin A, upon which diet they are kept until growth has ceased because of depletion of their bodily stores of vitamin A; then, leaving some on the basal diet only as negative controls, others are fed different fixed allowances of the food to be tested as their sole source of vitamin A until it is determined what daily allowance of the food under investigation

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The authors desire to record their indebtedness to Professors Jobling and Johnson of the Department of Pathology for guidance in the histological examinations; and to Miss L E. Booher and Miss H. C. Cameron for efficient collaboration in different parts of the experimental and analytical work

¹ Steenbock, H, and Nelson, E M, *J. Biol Chem*, 1923, lvi, 355.

² Drummond, J C, Coward, K H, and Handy, J, *Biochem. J.*, 1925, xix, 1068.

³ Sherman, H C, and Munsell, H. E., *J. Am. Chem. Soc.*, 1925, xlvii, 1639

is required to support an average rate of gain of 3 gm. per week during the experimental period in a standard test animal prepared as indicated. This daily allowance of food is then said to have furnished 1 unit of vitamin A. The method with a few modifications has been incorporated in the latest revision of the United States Pharmacopœia as an official procedure for the testing of cod liver oil for its vitamin A value. In order that such determinations of vitamin A shall be quantitatively uninfluenced by the vitamin D (or antirachitic) value of the material which is being tested for vitamin A, it is necessary that the vitamin D requirement of the test animal shall be provided for, at least to the extent needed for the maintenance of a rate of growth of 3 gm. per week. According to present knowledge, this antirachitic or vitamin D requirement may be met in any of four ways: by irradiation of the animal, or of its food, or the addition of irradiated cholesterol to the food, or by so feeding the families from which the test animals are drawn that they will have acquired bodily stores of vitamin D sufficient to meet their needs for the time and conditions of the vitamin A experiment when carried out as described.

The last named condition appears to have been fulfilled in most of the work of this laboratory upon the testing of foods for vitamin A even before Steenbock's discovery that the antirachitic factor is also an essential for growth

In two ways our animals were protected from such shortage of the antirachitic factor as seems to have inhibited growth in some of the cases described by Steenbock (1) Following the example of the English investigators, we greatly restrict the growth of the animal during the experimental period, in most cases to a gain of only about 3 gm. per week. The fact that very little growth is permitted by the vitamin A intake naturally has the effect of conserving such store of vitamin D as is contained in the body of the experimental animal. (2) This bodily store of vitamin D is doubtless made relatively larger in our animals than in Steenbock's by the difference in the diets of the families from which the young rats are drawn. In the diet used by Steenbock the vitamin A requirement is supplied chiefly by dried alfalfa leaves which, as his work shows, are poor in vitamin D; whereas in the diet chiefly used by us whole milk is the main source of vitamin A and this at the same time supplies vitamin D in relatively much more

liberal proportion than does the dried alfalfa, so that the animals raised on the diet in which vitamin A is supplied by whole milk undoubtedly acquire much larger bodily stores of vitamin D than do the animals raised on the diet in which the chief source of vitamin A is dried alfalfa.

Clinical reports of rickets occurring in milk-fed infants have apparently led to a general failure of appreciation of the antirachitic value of the fat of milk and butter. McCollum and his coworkers⁴ have included a small percentage of butter fat in their rickets-producing diet, but have recently pointed out that this limited amount of butter fat sometimes furnishes enough vitamin D to prevent the development of rickets; and Mellanby emphasizes the markedly favorable influence of butter fat upon the development and calcification of the bones of the puppies which he used as experimental animals in his studies of the rachitic and antirachitic effects of foods.⁵

In the work of Dr. Munsell in this laboratory it had been noted that among animals which had been equally retarded in growth by shortage of vitamin A, the bone development appeared somewhat better in those whose limited allowance of vitamin A had been furnished in the form of butter or whole milk than in those which had received the same limited amount of vitamin A in the form of certain other foods, notably carrots. From this it would appear that the most rigorous test of the question as to whether the method of Sherman and Munsell³ effects a quantitative differentiation between vitamins A and D would be afforded by feeding carrots as sole source of vitamin A (and sole immediate food source of vitamin D) and determining whether under these conditions the irradiation of a part of the experimental animals would increase their rate of gain in body weight; also, as a further step toward quantitative work with the antirachitic factor, we compared the gains in body calcium. As a further check upon results and conclusions there were also included in this work some additional experiments upon gain in weight with and without irradiation when the limited allowance of vitamin A was supplied by butterfat.

⁴ McCollum, E. V., Simmonds, N., Becker, J. E., and Shipley, P. G., *J. Biol. Chem.*, 1926, lxx, 437.

⁵ Mellanby, E., *Brit. Med. Research Council, Special Rep. Series, No. 93*, 1925, 24.

In a comparison of thirteen animals thus receiving their vitamin A in the form of butter fat with fourteen parallel cases fed in the same way but receiving ultra-violet irradiation in addition, the average gain in weight for the 8 weeks experimental period was 6 gm. less for the irradiated than for the non-irradiated animals. This confirms the view that shortage of vitamin D does not influence the weight curve obtained in the testing of foods such as butter for vitamin A by the method of Sherman and Munsell. It also indicates that for the purposes of the present investigation a difference of 6 gm. gain in body weight in the average of a group of a dozen or more of experimental animals should possibly be regarded as accidental.

TABLE I

Average Data of Body Weight and Body Calcium in Test Animals Receiving Limited Allowances of Vitamin A-Containing Food, without and with Ultra-Violet Irradiation

Sources of vitamins A and D	Gain in body weight		Final calcium content of body	
	No of cases	Average gm in 8 wks	No of cases	Average percentage of Ca
Carrot, 0.3 gm per wk	27	19	24	1.22
Same + irradiation	29	23	30	1.28
Carrot, 0.6 gm per wk	22	31	21	1.25
Same + irradiation	22	39	18	1.22

Table I summarizes the net effects, upon body weight and body calcium, of the 8 weeks of experimental feeding without and with ultra-violet irradiation. In order to economize space, only the averages for the principal series are tabulated. If the comparison is confined to cases of animals of similar size, the effect of irradiation upon the average gain in weight becomes more pronounced, *viz* 8 and 11 gm instead of 4 and 8 gm. respectively. This is probably significant.

Comparing the results of the two series in which butter and carrots respectively were fed, their chief significance would seem to be that (as suggested by Munsell) in the determination of vitamin A in butter fat (by the method used in this laboratory) there

is plainly no disturbing shortage of vitamin D, whereas in the determination of vitamin A in carrots by the same method there appears a possibility that in the absence of irradiation the weight curve employed as a means of measuring the amount of vitamin A supplied by the food may perhaps be influenced by a simultaneous shortage of vitamin D.

According to the broad definition of rickets used by Park⁶ and now generally accepted, and in the light of studies previously published from this laboratory upon the calcium⁷ and phosphorus⁸ contents of the body as influenced by age and development, it appeared not impossible that the calcium content of the body might throw additional light upon the problem in hand, especially if supplemented by studies of bone development in the experimental animals by means of x-ray photographs, line tests of the tibia, and histological examinations of the ribs.

Of fourteen irradiated animals whose knee joints were x-rayed, thirteen appeared normal and one slightly frayed; of six non-irradiated animals, one appeared normal, one slightly frayed, three showed slight rarefaction or blurring, and one showed a slightly broadened tibia.

Of ten line tests on irradiated animals, three were reported as moderate, five as good, two as very good; while of four such tests on non-irradiated animals, one was fair, two moderate, and one good.

Histological examinations of the rib junctions of the same fourteen cases which were submitted to the line test, showed only occasional doubtful or border line indications of possible departure from the normal histology, and these as often among the irradiated as among the non-irradiated animals.

Thus the x-ray, line test, and histological examinations, while not made in sufficiently large numbers of cases to justify any attempt at quantitative (statistical) interpretation, show mainly that there was no well marked difference in any of these respects between the irradiated and the non-irradiated animals; but that all these examinations taken together—and perhaps most clearly

⁶ Park, E A , *Physiol Rev* , 1923, iii, 1

⁷ Sherman, H C , and MacLeod, F. L., *J Biol. Chem* , 1925, lxiv, 429.

⁸ Sherman, H C , and Quinn, E J , *J Biol. Chem* , 1926, lxxvii, 667.

the general trend of the line tests—may be taken as affording a slight indication of shortage of vitamin D in the cases in which carrot served as the sole food source of fat-soluble vitamin and no irradiation was supplied. Turning to the last two columns of Table I to see whether this qualitative impression is borne out by the quantitative determinations of body calcium we find that, compared in groups of eighteen to thirty animals each, the average percentage of body calcium seems in the first series to have been slightly increased by the irradiation; but that in the second series there is as large an average difference in the opposite direction, which can only be interpreted as due to chance or physiological variability of the animals.

Any attempt to compare the data here found with strictly normal values for body calcium is complicated by the fact that the animals of all four of the groups here considered had been inhibited in growth by the shortages of vitamin A in their food. In all of these cases, as in similar cases previously studied in this laboratory⁷ it was found that the amount of calcium in the body of an animal whose growth had been thus retarded was between the normal for the age and the normal for the weight. As has been indicated, the present data are especially significant as affording a direct comparison of animals with and without irradiation, when in both cases the restriction of vitamin A intake had been exactly the same.

Factor for Estimating the Total Calcium Content of the Body from the Calcium Content of the Two Femurs.—The direct determination of the total calcium of the body is not possible if ribs, tibia, or other parts of the animal are used for other types of test, yet data of both types for the same animals are often desirable. It was therefore decided in connection with this investigation to establish a factor by means of which, from a determination of the amount of calcium found in the two femurs, the calcium content of the entire body can be calculated. 56 determinations of femur calcium and of the total body calcium of the same animals were used for the establishment of this factor. The mean value found was 14.138 with a probable error of ± 0.135 , a standard deviation of 1.500, and a coefficient of variation of 10.6 per cent. The limits of value for this factor which would cover a range of 4 times the probable error of the mean are, therefore, 13.60 to 14.65. In investigations such

as those here described, a convenient approximation of the total calcium content of the body may be obtained by multiplying the femur calcium by 14.14.

The 56 cases from which this average is derived were all of young rats 80 to 120 days old, whose growth had been inhibited in varying degrees by shortages of vitamin A. The ratio of femur calcium to total body calcium in rats of different ages and nutritional histories is also being investigated in this laboratory.

CONCLUSIONS.

1. In experiments carried out under the conditions of the method previously reported from this laboratory for the quantitative determination of vitamin A in foods, it has been found that irradiation of the test animals with the mercury vapor quartz lamp does not produce any large increases in the growth of animals receiving limited amounts of vitamin A. This may be explained on the ground that our animals contain, on the average, a nearly sufficient bodily store of antirachitic vitamin to carry them through the test period where growth is limited as in our method. This is confirmed by a study of the bone structure, which showed few and minor deviations from the normal x-ray picture, line test, and histological appearance. Under the conditions of our experiments, the weight curve employed as a measure of the vitamin A content of the food under investigation was not influenced by shortage of vitamin D when the food was butter fat; but was somewhat influenced when the food was carrot. Evidently the butter fat contained more vitamin D in relation to its vitamin A content than did the carrot. Unless both the character of the food and the bodily store of vitamin D in the test animals have been established by previous experimentation, irradiation or the feeding of irradiated food to ensure an adequate supply of vitamin D is a wise precaution. The extent of the difference which this may make varies widely with the stock diets used in different laboratories.

2. The animals which had received their limited allowance of vitamin A in a form which, as the result of previous work, was judged most likely to involve a shortage of vitamin D, were investigated further by quantitative determinations of body calcium. The results do not justify a positive conclusion as to whether or

not the irradiation influenced the percentage of calcium in the body in these cases. In one series of experiments, irradiation appeared to result in a slight increase in the percentage of body calcium; but in a second series this result was not confirmed. It is possible that the degree of calcification in animals which had been for about 12 weeks on a diet that markedly retards growth, is less delicate than would be a test for calcification at an earlier stage, and perhaps it may be a less quantitative indication of a shortage of vitamin D than the weight curve under adequately controlled conditions. Further studies of this question are being undertaken.

3. In experimental animals (rats) such as those here described, the factor by which femur calcium could be multiplied to obtain total body calcium was, in the mean of 56 cases, 14.14.

NOTE ON A PORTABLE FORM OF THE MANOMETRIC GAS APPARATUS, AND ON CERTAIN POINTS IN THE TECHNIQUE OF ITS USE.

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(Received for publication, February 21, 1927)

The form of the manometric apparatus shown in Figs. 1 and 2 is more convenient in several respects than the original model described by Van Slyke and Neill (1). The chief convenience is that in the present model all parts, including motor and shaking device, are attached to one simple, compact, rigid, wooden frame, so that the apparatus can be packed, shipped, and moved about from desk to desk as a unit. It is attached firmly to any table top by a carpenter's screw clamp, shown in Fig. 1.¹

The strong, rigidly attached iron rod to which the pulley is clamped reduces vibration to a minimum and assists in making a noiseless, smooth-running mechanism.

The prolongation of the glass tubing for the mercury below the level of the table top is rendered unnecessary by the use of the closed manometer

As in the original, it will be noted that the bore of the glass manometric tubing is constricted to 1 mm at one point above and one point below the scale. The upper constriction is to prevent mercury from striking the upper cock with destructive force, the lower one is to diminish the tendency of mercury to oscillate in the manometer while the gas volume is being reduced to the 2 or 0.5 cc. mark in the chamber.

The bottle on top of the frame holds distilled water. The lower bottle is to receive waste solutions drained out of the chamber

¹ The apparatus is manufactured by Eimer and Amend of New York, the Arthur H. Thomas Company of Philadelphia, the Empire Laboratory Company of New York, and by Robert Goetze, of Leipsic

after analyses. The most rapid and convenient way to transfer them to the waste bottle is to force them up into the cup above the chamber and draw them over into the bottle by suction, arranged as indicated in Fig. 1. If suction is not available, how-

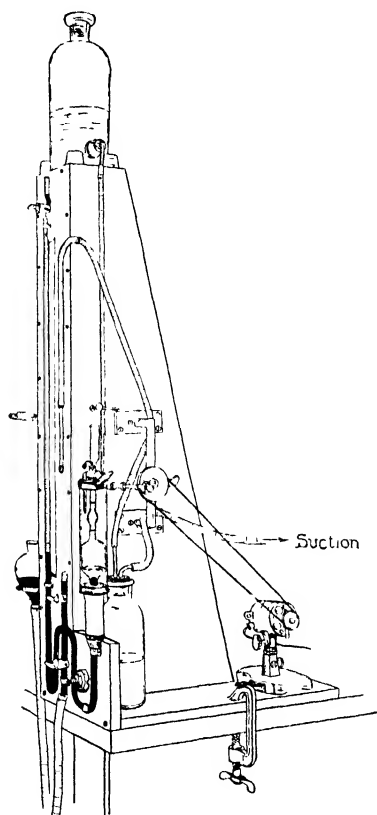
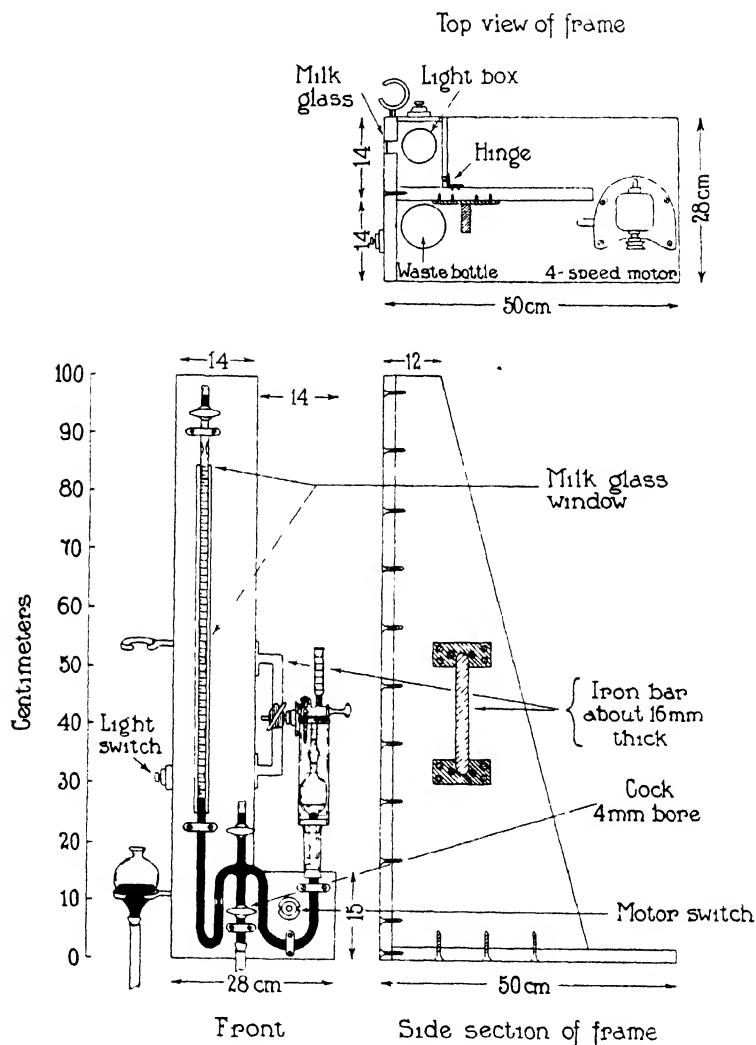


FIG. 1.

ever, a narrow rubber drain tube can be run directly from the curved outlet capillary of the chamber to the waste bottle.

The dimensions given in the scale drawing of Fig. 2 should be adhered to. In particular the + joint in the center tubing must be located at the level indicated.

The rubber tubing for connecting the mercury-leveling bulb,



and for making the inner joint connecting the gas chamber with the glass tube from the manometer (for details of joint see Fig. 2 of Van Slyke and Neill) must be the *heavy walled red nitrometer*

tubing. Ordinary rubber leaks air, and also dirties the mercury with sulfide, which in turn pollutes the manometer.

The metal parts must all be of iron or steel. Brass is destroyed by the contact with mercury which is certain to occur.

As a *dehydrating agent* to wet the upper part of the manometer tube we have replaced the concentrated sulfuric acid, used by Van Slyke and Neill, by glycerol, by Kahlbaum's diethylene or trimethylene glycol, or by the methylene glycol sold as "anti-freeze" for automobile radiators. These organic fluids have the advantage that they do not char the lubricant used on the cock above the manometer. The methylene and ethylene glycols are less viscous, and preferable to glycerol for that reason. Once every few days about 1 cc. is admitted into the part of the manometer tube immediately below the cock, and is then ejected. The portion that remains adherent on the walls is sufficient to absorb the vapor of moisture drawn into the manometer tube by the mercury.

Absorption of Oxygen—For absorption of oxygen in the gas chamber the catalyst, sodium anthrahydroquinone- β -sulfonate, introduced by Fieser (2), added to the hydrosulfite, markedly accelerates the reaction.² We grind up in a mortar 100 gm. of sodium hydrosulfite and 10 gm. of β -sulfonate, and keep the mixture in a stoppered bottle. For preparation of the absorbent solution 10 gm. of this mixture are placed in a beaker, and 50 cc. of 1 N potassium hydroxide are poured over it. The mixture is stirred with a rod for a few seconds and quickly filtered through cotton. The filtrate is at once deaerated in the gas apparatus as described on pp. 534 and 535 of Van Slyke and Neill's paper. As noted by E. K. Marshall (personal communication) a drop of 10 per cent ferric chloride added to the solution still further accelerates its activity.

Absorption of oxygen by this solution is almost as rapid as absorption of CO_2 by alkali if the following procedure is followed: After the p_1 reading of $\text{O}_2 + \text{N}_2$ has been taken the solution in the chamber of the apparatus is lowered until a gas space of 4 or 5 cc. is obtained. The cock leading to the mercury bulb is then closed. 1.5 cc. of hydrosulfite solution are placed in the cup of

² The sulfonate is obtainable from the Eastman Kodak Co.

the apparatus, and admitted a drop at a time. As each drop trickles down the inner wall of the chamber it absorbs oxygen, and the mercury in the manometer falls. After a few drops have been admitted no further perceptible fall occurs. The mercury cock is then opened, and the solution in the chamber is permitted to rise as near to the stop-cock as it will, with the leveling bulb in

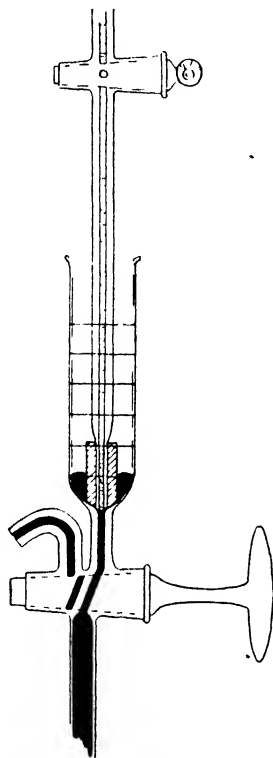


FIG. 3

the position shown in Figs. 1 and 2. The remainder of 1 cc. of hydrosulfite is then added, completing the absorption of the last traces of oxygen. The entire absorption can be completed in about 1 minute with the gas from 2 cc. of blood, and in considerably less with the gas from 1 cc.

For micro determinations, with 0.2 cc. portions of blood, the

preliminary absorption under reduced pressure is dispensed with. A few drops of absorbent, added with the mercury cock open and the leveling bulb in the position shown in Figs 1 and 2, complete the absorption in a few seconds

Quantitative Transfer of Solution to the Chamber without Washing —With a mercury seal in the cup, solutions can be pipetted directly into the chamber of the apparatus. A pipette is used calibrated to deliver between two marks, and preferably provided with a stop-cock, as shown in Fig 3. From 0.5 to 1.0 cc. of mercury is forced up into the cup, and the rubber-tipped point of the pipette (see p 532 of Van Slyke and Neill's paper) is immersed in the mercury and fitted accurately into the bottom of the cup. The flow of solution from the pipette into the chamber may be regulated by either the cock of the pipette or that of the chamber.

After the pipette is withdrawn the mercury is admitted into the chamber, forcing through before it the solution in the capillary. A slight loss occurs, due to the fact that a droplet of solution from the top of the capillary rises to the top of the mercury when the pipette is withdrawn. We have determined this loss by delivering 5 cc. of 1 N sodium hydroxide into the chamber by the above technique, then pouring 2 or 3 cc. of water into the cup, washing the mercury about with it, and titrating the alkali with 0.02 N acid, using alizarin sulfonate as indicator. The amount of 0.02 N acid used was 0.25 to 0.35 cc., equivalent to 0.005 to 0.007 cc. of the 1 N alkali. With properly fitting pipette tip and rubber ring, therefore, the loss in the above method of delivery can be kept within these limits. The technique can accordingly be used for the quantitative transfer of portions of 2 cc. or greater volume, without significant loss. For quantitative transfer of smaller portions washing must be used.

The portable apparatus above described was developed with the assistance of John Plazin. A marked mechanical improvement in the form of a well machined ball bearing pulley, reducing vibration to a minimum, was introduced by Mr. Walter Eimer of the firm of Eimer and Amend.

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CARBON DIOXIDE FACTORS FOR THE MANOMETRIC BLOOD GAS APPARATUS.

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When CO_2 is determined in either the original volumetric apparatus of Van Slyke or the present manometric apparatus the gas is extracted from solution by shaking the latter in the evacuated chamber until equilibrium between gas phase and liquid phase is reached. Mercury is then admitted into the chamber until the gas phase is reduced from 43 or more cc. to 2 cc. or less before the gas is measured. During this reduction of volume the tension of CO_2 in the gas phase is proportionately increased, and in consequence a small part of the CO_2 passes back into the solution. Van Slyke and Stadie (1921) found that this reabsorption under the conditions of analysis in the volumetric apparatus amounted quite constantly to 1.7 per cent of the total CO_2 present in the system, and to correct for it introduced the α factor 1.017 into their calculation factors. Van Slyke and Neill (1924) for the manometric apparatus found an α factor of 1.014 when the gas phase in the 50 cc apparatus was reduced to 2 cc., 1.03 when it was reduced to 0.5 cc

An increasing refinement of technique in the preparation, handling, and analysis of standard carbonate solutions has made it possible to determine the α factor for the manometric apparatus with increased precision. Both direct determinations of reabsorbed CO_2 , and estimations of it from the proportion of expected CO_2 obtained, indicate that for the above conditions the α factor has the values 1.017 and 1.037 instead of the former values 1.014 and 1.030.

Furthermore our results indicate that the value 22.26 liters, determined by Guye and Pintza (1908) for the molecular volume

of CO_2 measured at 0° , 760 mm pressure, is exact, rather than the conventional 22.4 liters. Van Slyke and Neill employed the value 22.4 in their calculations, and intentionally incorporated any inaccuracy due to the use of this figure in their α factors. The total effect of changing the α factor from 1.014 to 1.017 and the molecular volume from 22.40 to 22.26 liters is to increase by approximately 1 per cent the factors of Van Slyke and Neill for calculating millimolar CO_2 concentrations. Volume per cent factors are affected only by the change in α , and are increased by but 0.3 per cent.

The results of analyses of standard sodium carbonate solutions, on which the work in this paper is based, have been further verified by determinations of the CO_2 evolved by permanganate oxidation of oxalic acid in connection with gasometric calcium analyses (Van Slyke and Sendroy, 1926). The authors have checked each others results. In this paper the results with carbonate solutions only will be presented.

Calculations.

The fundamental equation, on which all calculations in the manometric gas apparatus are based, is the following (Equation 4 of Van Slyke and Neill):

$$(1) \quad V_{0^\circ, 760} = a \times \frac{P}{760} \times \frac{1}{1 + 0.00384 t} \times \left(1 + \frac{S}{A - S} \alpha'\right) \times \alpha$$

Observed gas volume.	Correction for pressure	Correction for temperature effect on gas and on Hg in manometer	Correction for unextracted gas, according to Henry's law	Correction for reabsorbed gas
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We may combine all the factors except P into a single factor, by which P is multiplied to obtain $V_{0^\circ, 760}$. Such a combined factor, with given a , A , and S , is dependent only on the temperature

$$(2) \quad V_{0^\circ, 760} = P \times \left[\frac{\alpha a}{760 (1 + 0.00384 t)} \times \left(1 + \frac{S}{A - S} \alpha'\right) \right]$$

$$= P \times [\text{volume factor}]$$

$V_{0^\circ, 760}$ = volume of gas, measured at 0° , 760 mm, in the solution analyzed; P = partial pressure of the extracted gas read on the

manometer; ι = the correction factor for reabsorption; a = the volume to which the gas phase is reduced after extraction and at which the final pressure readings are made, t = temperature in °C.; S = total volume of solution from which the gas is extracted; A = the volume of chamber occupied by gas and solution during extraction; $A - S$ = volume of gas phase during extraction; α' is the Henry distribution coefficient of the gas between gas and liquid phases $\alpha' = \alpha \times \frac{T}{273}$, where α is the Bunsen solubility coefficient, T the absolute temperature. For illustration of the values a , A , S , $A - S$, see Fig 1 of Van Slyke and Neill.

Dividing the volume factor in Equation 1 by 22.26, the volume in cc. of 1 milligram molecule (= 1 millimol = 44 mg.) of CO_2 reduced to 0°, 760 mm., we have

$$(3) \quad \text{mm CO}_2 \text{ in the portion of solution analyzed} \\ = P \times \left[\frac{\iota a}{16,918 (1 + 0.00384 t)} \times \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

Multiplying the volume factor in Equation 2 by 1.9766, the weight in mg. of CO_2 at 0°, 760 mm., we have

$$(4) \quad \text{Mg CO}_2 = P \times \left[\frac{1.9766 \iota a}{760 (1 + 0.00384 t)} \times \left(1 + \frac{S}{A - S} \alpha' \right) \right]$$

To change to terms indicating CO_2 as volumes per cent or millimols per liter in the solution analyzed the factors in Equations 2 and 3 are multiplied by $\frac{100}{\text{cc sample}}$ and $\frac{1000}{\text{cc. sample}}$, respectively, and yield the following:

$$(5) \quad \text{Vol per cent CO}_2 = P \times \left[\frac{0.13159 \iota a}{(\text{cc sample}) (1 + 0.00384 t)} \times \left(1 + \frac{S}{A - S} \alpha' \right) \right] \\ = P \times \text{vol per cent factor}$$

and

$$(6) \quad \text{mm CO}_2 \text{ per liter} = P \times \left[\frac{0.05911 \iota a}{(\text{cc sample}) (1 + 0.00384 t)} \times \left(1 + \frac{S}{A - S} \alpha' \right) \right] \\ = P \times \text{mm per liter factor}$$

The observed reading, P , is multiplied in each case by a total factor (in brackets) to calculate in the terms desired the amount

of CO_2 present in the sample of solution analyzed (by Equations 1 to 4), or the concentration of CO_2 , in volumes per cent or millimols per liter, in the solution from which the sample for analysis was taken (Equations 5 and 6).

The factor i was determined by direct estimation, in a way described later, and was also calculated from analyses of standard solutions of Na_2CO_3 according to Equation 7, which is Equation 8 of Van Slyke and Neill with the factor 0.05911 in place of 0.0587.

$$(7) \quad i = \frac{1}{P} \times \frac{[\text{CO}_2] \text{ (cc sample)}}{0.05911 a} \times \frac{1 + 0.00384 t}{1 + \frac{S}{A - S} \alpha'}$$

$[\text{CO}_2]$ represents millimols of CO_2 present per liter of the standard solution.

EXPERIMENTAL.

Preparation of Standard Na_2CO_3 Solutions.

The purest NaHCO_3 obtainable was heated in a furnace for 3 hours at a temperature of $290\text{--}300^\circ\text{C}$. After slight cooling, the resultant Na_2CO_3 was placed in a desiccator in a bottle, which was opened only when a solution was to be prepared.

The water used to prepare the standard solutions was redistilled from alkaline or acid potassium permanganate and received in flasks protected from atmospheric CO_2 by soda-lime tubes. The distillate, however, always contained several hundredths of a volume per cent of CO_2 . The preparation and preservation of really CO_2 -free water is, in fact, impossible without extraordinary precautions. Instead of attempting it we have in each blank analysis of the reagents substituted for the sodium carbonate solution an equal volume of the water with which it was prepared, so that the result of the blank included the corrections both for the reagents and for the CO_2 in the water. The $P\text{CO}_2$ readings observed in the blanks were subtracted from those obtained in analyses of the standard carbonate solutions.

The carbonate solutions, as soon as prepared, were transferred to Pyrex glass tubes described by Austin *et al.* (1922), and kept over mercury and out of contact with the atmosphere. In this condition they could be kept for days without change in CO_2 content. The portions for analysis were drawn into stop-cock

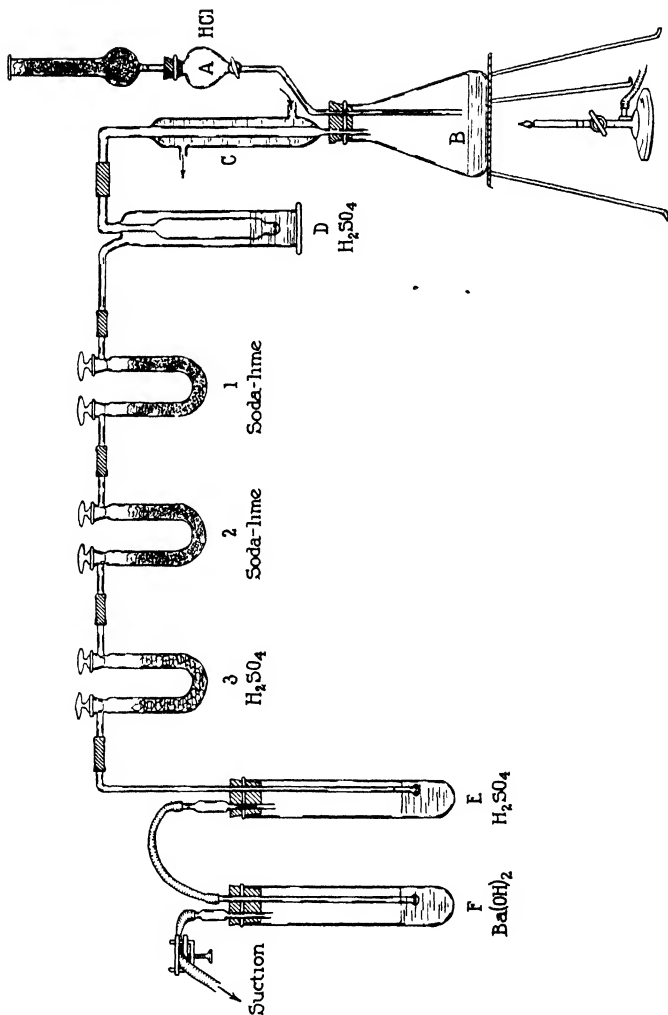


FIG 1

pipettes as described under "Sampling Blood" on p. 131 of the Austin *et al.* paper.

In order to control the purity of the carbonate and the accuracy of the standard solutions, they were analyzed as described below, by two gravimetric and one titrimetric method.

1. Carbon dioxide in both solid Na_2CO_3 and standard Na_2CO_3 solutions was determined gravimetrically by the well known method of driving the gas out of solution by acidifying, heating, and passing a stream of CO_2 -free air. The evolved CO_2 was absorbed and weighed in soda-lime tubes

A known amount of standard solution, or of solid carbonate and analyzed water, was placed in the flask *B* (Fig. 1), which was then connected with the rest of the closed system as indicated. A protecting soda-lime tube was attached to the funnel, *A*, which dropped dilute HCl into the flask. The evolved CO_2 passed through the condenser, *C*, the wash bottle, *D*, containing concentrated sulfuric acid, two weighed U-tubes containing soda-lime (1 and 2), and a third containing pumice stone saturated with sulfuric acid (3), then through the aeration tubes, *E* and *F*, containing sulfuric acid and clear barium hydroxide solution, respectively. A slow current of air was drawn through the train by suction, the evolution of gas being regulated by means of the stop-cocks on the funnel and the pinch-clamp on the suction tube. After addition of the acid heat was applied to the flask, and the temperature of the solution was very slowly increased to boiling. The stem of the funnel was then lowered beneath the surface of the boiling liquid, and air was drawn through the system until all the CO_2 was transferred to the soda-lime tubes. The barium hydroxide solution at the end of the train indicated any escape of CO_2 . When this occurred the analysis was discarded. The three U-tubes were carefully cleaned with ether, wiped dry with lens paper, and allowed to come to room temperature before each weighing. Blank analyses done on the same day, on the same water used for the solution, served to correct for the amount of CO_2 in the water and in the air of the flask and wash bottle.

Some typical results are given in Table I.

2. To determine the sodium contents, portions of the same solutions of Na_2CO_3 were measured out into platinum dishes and dilute H_2SO_4 was added, with care to avoid spattering. The solu-

tions were concentrated as far as possible on the water bath and the free H_2SO_4 was driven off by heating, first on a hot plate, and finally with a micro burner. The sodium sulfate was dissolved in water, and any excess H_2SO_4 not driven off was detected and measured by titration with standard NaOH solution. The solu-

TABLE I

Gravimetric Determination of CO_2 in Standard Sodium Carbonate Solution.
Solution contained 10 5753 gm Na_2CO_3 per liter

Volume of sample	Calculated weight of CO_2 in sample	Weight of CO_2 found minus average blank of 0 0006 gm (3 analyses)	Per cent of theoretical
cc	gm	gm	
100	0 4390	0 4392	100 05
100	0 4390	0 4385	99 89
100	0 4390	0 4389	99 98
Average			99 97

TABLE II

Gravimetric Determination of Sodium in Standard Sodium Carbonate Solution as Barium Sulfate

Na_2CO_3 in solution	a BaSO_4 found	0 1 N NaOH to neutralize free H_2SO_4	b BaSO_4 equivalent to free H_2SO_4	a - b BaSO_4 from Na_2CO_3	Na found in Na_2CO_3	Per cent of theoretical Na found.
gm	gm	cc	gm	gm	per cent	
0 2115	0 4728	0 57	0 0067	0 4661	43 42	100 07
0 2115	0 4741	0 67	0 0078	0 4663	43 44	100 11
0 2027	0 4944	4 09	0 0477	0 4467	43 42	100 07
0 2027	0 4949	3 91	0 0456	0 4473	43 48	100 20
Average					43 44	100 11
Theoretical					43 39	

tions were then acidified with HCl , and the sulfate was precipitated hot with BaCl_2 . Table II indicates the results obtained.

3. As a further check on the accuracy of the standard solutions, the latter were also titrated. An excess of 0.1 N HCl made from constant boiling acid prepared according to Hulett and Bonner

(1909) was added to the carbonate solution, which was then boiled to drive off CO_2 . After cooling, the excess HCl was titrated back with 0.1 N NaOH which had been standardized against the same HCl solution. Various analyses at different times and on different solutions indicated a content of Na_2CO_3 varying between 99.7 per cent and 100.2 per cent of the theoretical.

TABLE III

Determination of Reabsorption Correction, r , by Analysis of a Standard Na_2CO_3 Solution

$A = 100.0$ cc $S = 7.00$ cc $a = 4.009$ cc 2 cc samples of Na_2CO_3 solution
Blank = 2.0 mm = c

Solution	Na ₂ CO ₃ concentration [CO ₂]	<i>p</i> ₁	<i>p</i> ₂	<i>P</i> CO ₂ = <i>p</i> ₁ - <i>p</i> ₂ - <i>c</i>	Tempera- ture	¹ Calculated by Equa- tion 7	Deviation from aver- age of Tables III and IV
	<i>mM</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	°C		
A	49.89	652.3	231.0	419.3	21.9	1.020	+0.003
		651.5	231.0	418.5	21.0	1.017	0.000
		650.6	230.8	417.8	20.7	1.017	0.000
		651.4	232.0	417.4	21.0	1.020	+0.003
		655.1	234.0	419.1	21.8	1.020	+0.003
		658.0	235.0	421.0	22.4	1.018	+0.001
		657.4	234.8	420.6	22.0	1.017	0.000
		656.6	234.6	420.0	22.0	1.019	+0.002
		658.5	235.1	421.4	22.2	1.016	-0.001
		660.0	237.0	421.0	22.8	1.020	+0.003
		660.7	236.6	422.1	23.0	1.019	+0.002
		663.8	237.6	424.2	23.8	1.018	+0.001
		666.7	238.8	425.9	24.3	1.016	-0.001
3 cc samples of Na ₂ CO ₃ solution Blank = 1.8 mm = <i>c</i>							
E	33.26	688.6	259.6	426.5	24.8	1.017	0.000
		688.5	258.5	427.5	24.8	1.015	-0.002
		688.5	259.0	427.0	24.8	1.016	-0.001

From the foregoing, it is apparent that the Na_2CO_3 used was as nearly pure as could be ascertained by methods approaching one per thousand in accuracy, and hence was a proper basis for standard solutions.

Determination of the ι Factor.

To determine the ι factor, standard sodium carbonate solutions of varying concentration were analyzed by the Van Slyke-Neill technique during the course of 4 months in several different blood gas apparatus, of different capacity, with open and closed manometers. From the results the ι correction was estimated

TABLE IV

Determination of Reabsorption Correction, ι , by Analysis of a Standard Na_2CO_3 Solution.

$A = 50$ cc $S = 3.5$ cc $a = 2.002$ cc 1 cc. samples of Na_2CO_3 solution.
Blank = 0.7 mm

Solution	Na_2CO_3 concentration [CO ₂]	p_1	p_2	$P_{\text{CO}_2} =$ $p_1 - p_2 - 0.7$	Temperature	ι Calculated by Equation 7	Deviation from average of Tables III and IV
	mm	mm	mm	mm	°C		
B	49.99	492.0	71.5	419.8	21.2	1.018	+0.001
		491.6	72.0	418.9	21.2	1.020	+0.003
		491.4	71.3	419.4	21.2	1.019	+0.002
		491.6	71.2	419.7	21.3	1.019	+0.002
		492.5	71.8	420.0	21.4	1.019	+0.002
		491.5	71.4	419.4	21.2	1.019	+0.002
C	15.00	201.4	73.5	127.2	22.5	1.015	-0.002
		201.1	73.3	127.1	22.7	1.017	0.000
		202.2	74.0	127.5	23.0	1.016	-0.001
		202.3	74.0	127.6	23.2	1.016	-0.001
		202.4	74.0	127.7	23.2	1.015	-0.002
D	30.00	336.9	83.4	252.8	21.5	1.016	-0.001
		337.7	84.0	253.0	21.5	1.015	-0.002
		336.3	83.5	252.1	21.1	1.017	0.000
		336.4	83.5	252.2	20.8	1.015	-0.002

by Equation 7. Within the limits of error, the same results were given by all the different apparatus.

The technique followed for reducing the gas volume from $(A - S)$ to a cc. was that described on p. 533 of Van Slyke and Neill's paper. The mercury cock was opened with a smooth, gradual motion, the mercury was permitted to rise fairly rapidly through

the wide, cylindrical part of the chamber, and was gradually retarded as the meniscus of the solution approached the *a* mark. When the latter was reached the movement had been so retarded that *no oscillation* of the solution in the chamber occurred after the mercury cock was closed. As pointed out on p. 26 of Van Slyke and Stadie's paper, such oscillation can cause a gross increase in reabsorption of CO_2 . The time taken for the volume reduction was 30 to 40 seconds in the 50 cc. apparatus, 45 to 55 seconds in the 100 cc. apparatus. If much more time is taken, reabsorption is increased. However, it is our experience that an

TABLE V

Determination of Reabsorption Correction, ι , by Analysis of a Standard Na_2CO_3 Solution under Conditions for Micro Analysis in 100 Cc Apparatus with $a = 1$ Cc

$A = 100$ cc $S = 7.00$ cc $a = 1.000$ cc 3 cc samples of Na_2CO_3 solution
Blank = 6.4 mm

Solution	Na_2CO_3 (concentration [CO_2])	p_1	p_2	$P_{\text{CO}_2} =$ $p_1 - p_2 - 6.4$ blank	Temperature	ι Calculated by Equation 7	Deviation from average of Tables V and VI
	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	$^{\circ}\text{C}$		
M	8.04	697.2	287.5	403.3	23.2	1.034	-0.003
		696.5	287.9	402.2	23.4	1.038	+0.001
		699.6	208.7	404.5	23.7	1.034	-0.003
		696.3	285.5	404.4	23.8	1.035	-0.002
		695.1	286.4	402.3	23.7	1.040	+0.003
		694.8	285.3	403.1	23.5	1.036	-0.001
		694.3	284.5	403.4	23.6	1.036	-0.001
		644.1	283.4	403.8	23.7	1.036	-0.001

analyst, after acquiring facility in the use of the apparatus, automatically learns to admit the mercury at rates within the necessary limits. The p_1 manometer reading is made as soon as convenient after the gas has been brought to *a* cc volume. The meniscus will stand thus for some seconds without measurable effect on p_1 , but longer standing results in significant increase in reabsorption.

Some typical analyses are given in Tables III to VI. The average of the results in Tables III and IV gives a value of 1.017 for

i when readings are made with a gas volume 2.0 cc. in the 50 cc. apparatus or of 4.0 cc. in the 100 cc. apparatus. Tables V and VI indicate an average value of 1.037 for i when readings are made in the 50 cc. apparatus with a gas volume of 0.5 cc., or in the 100 cc. apparatus with a gas volume of 1.0 cc.

In order further to verify these i factors the following method was employed to obtain a more direct determination of the reab-

TABLE VI

Determination of Reabsorption Correction, i , by Analysis of a Standard Na_2CO_3 Solution under Conditions for Micro Analysis in 50 Cc. Apparatus with $a = 0.5$ Cc.

$A = 50$ cc $S = 2.0$ cc $a = 0.500$ cc 1 cc samples of Na_2CO_3 solution.
Blank = 3.7 mm

Solution	Na_2CO_3 concentration [CO ₂]	p_1	p_2	$P_{\text{CO}_2} =$ $p_1 - p_2 - 3.7$	Temperature	i Calculated by Equation 7	Deviation from average of Tables V and VI
	<i>mM</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>°C</i>		
F	7.50	382.0	122.7	255.6	22.5	1.040	+0.003
		379.8	120.0	256.1	22.5	1.038	+0.001
		382.1	121.2	257.2	22.7	1.035	-0.002
		381.3	121.8	255.8	23.0	1.042	+0.005
		382.9	121.4	257.8	22.9	1.033	-0.004
		381.2	120.5	257.0	23.0	1.037	0.000
G		368.9	108.9	256.3	21.7	1.034	-0.003
		366.9	107.3	255.9	21.8	1.036	-0.001
		366.2	107.3	255.2	21.7	1.038	+0.001
		368.3	108.0	256.6	22.6	1.037	0.000
		365.5	105.5	256.3	23.0	1.040	+0.003
		369.6	107.8	258.1	23.1	1.033	-0.004

sorbed CO₂. With the Harington-Van Slyke (1924) apparatus, a standard solution of Na_2CO_3 was analyzed in such a way as to determine (a) the amount of CO₂ left in solution after the first extraction and attainment of equilibrium, (b) this amount of CO₂ plus the amount reabsorbed during release of the vacuum and reduction of the gas to the volume at which the pressure was read. The difference between (a) and (b) gives the amount of CO₂ reabsorbed.

The quantity (a) was determined as follows: 0.15 cc. of 10 per cent lactic acid and 2.35 cc. of water were placed in the chamber of a 50 cc. Harington-Van Slyke apparatus and freed of all gases by twice extracting the solution and ejecting the gases. About 1 cc. of the extracted solution was forced up into the cup, and a 1 cc. sample of standard carbonate solution was run from a stop-cock pipette into the chamber of the apparatus, with the technique detailed on p. 532 of Van Slyke and Neill's paper. The acid solution in the cup was returned to the chamber, the latter was evacuated, and the CO_2 was extracted. With the lower stop-cock of the Harington-Van Slyke chamber closed, the upper stop-cock was opened, and air was allowed to enter to atmospheric pressure. The CO_2 gas in the chamber was thus diluted with air without changing the CO_2 tension from that obtained at equilibrium at the end of the extraction. Mercury was then admitted into the chamber through the lower cock till all the air- CO_2 gas mixture was expelled through the upper cock, the solution being retained in the chamber. In this manner the CO_2 tension of the gas phase above the solution was kept constant, so that there was no reabsorption of CO_2 during ejection of the gases. The solution was again extracted, and the pressure was read at the 2.0 or the 0.5 cc. mark, giving p_1 . The CO_2 was then absorbed with 2 drops of 20 per cent NaOH , and the reading p_2 was made. Using the factors for a 1 cc. sample in Table X of this paper, the unextracted CO_2 was calculated, in terms of millimols per liter of the original sample analyzed. Table VII shows the good agreement obtained between the CO_2 thus determined and that calculated to be left in the solution after the first extraction, thereby confirming the validity of Henry's law under the conditions of the analysis. The calculated unextracted CO_2 values in the last column of the table were obtained by multiplying the original CO_2 content of the solution analyzed, *viz.* 30.08 mm., by the factor $\frac{S}{A - S} \alpha'$.

For the direct determination of (b), *unextracted plus reabsorbed* CO_2 , the technique of the usual CO_2 determined was carried through up to the point where the meniscus of the extracted solution was raised to the 2 cc. mark in the Harington-Van Slyke chamber. The lower stop-cock of the chamber was then quickly

closed, and a suction tube attached to a pump was pressed into the bottom of the cup. The upper stop-cock was then opened, whereupon the suction immediately drew the gas out of the space above the solution in the chamber. By repeatedly pressing the suction tube into the bottom of the cup for a few seconds and then removing it, the replacement of the CO_2 in the gas space of the chamber by air was made complete. Since the lower cock of the

TABLE VII
Direct Determination of Unextracted CO_2 in a Standard Sodium Carbonate Solution

Solution	p_1	p_2	$P_{\text{CO}_2} = p_1 - p_2$	Temperature	Unextracted CO_2 *	
					Observed	Calculated $\frac{as}{A-s}\alpha'$

$A = 50$ cc $S = 3.5$ cc $a = 2.003$ cc 1 cc samples of Na_2CO_3 solution Concentration = 30.08 mm per liter, including CO_2 content of water

	mm	mm	mm	°C	mm	mm
H	182.9	166.3	16.6	23.3	1.95	1.96
	182.3	166.1	16.1	25.0	1.88	1.88
	187.0	171.0	16.0	24.9	1.87	1.88
	181.2	165.8	15.4	24.9	1.80	1.88
	181.6	165.3	16.3	24.6	1.91	1.90
	179.6	162.1	17.5	20.8	2.09	2.09

$A = 50$ cc $S = 3.5$ cc $a = 0.500$ cc 1 cc samples of Na_2CO_3 solution Concentration = 40.00 mm per liter including CO_2 content of water

	mm	mm	mm	°C	mm	mm
J	300.3	214.2	86.1	23.8	2.57	2.58
	300.3	216.0	84.3	24.0	2.52	2.56
	288.0	203.0	85.0	23.8	2.54	2.58

* In mm per liter of original solution.

chamber was closed, the meniscus of the solution in the narrow tube of the chamber at the 2.0 cc. mark remained perfectly quiet, and there was no measurable loss of previously unextracted CO_2 from the solution. Finally the air was expelled from the chamber by admission of mercury from below, and the CO_2 remaining in the solution was determined in the usual manner, by extracting, reducing the volume to 2.0 cc, and measuring the pressure before

and after absorption of the CO_2 gas with 2 drops of 20 per cent NaOH . The reabsorbed CO_2 was computed as the difference between the calculated volume value of (a) and the determined value of (b), the accuracy of the factor $\frac{S}{A-S}$ for calculating (a) having been demonstrated by the data in Table VII. The

TABLE VIII
Direct Determination of Reabsorbed CO_2 in a Standard Sodium Carbonate Solution

Solution.	p_1	p_2	$P_{\text{CO}_2} = p_1 - p_2 - c$	Temperature	a CO_2 unextracted + reabsorbed	b CO_2 unextracted calculated as $30.08 \times \frac{S}{A-S} \alpha'$	$a - b$ CO_2 reabsorbed	Proportion of total CO_2 reabsorbed	Deviation from average
50 cc $S = 3.5$ cc $a = 2.003$ cc 1 cc samples of Na_2CO_3 solution Concentration = 30.08 mm per liter including CO_2 content of water.									
K	mm	mm	mm	$^{\circ}\text{C}$	mm	mm	mm		
	185.3	163.9	21.4	24.3	2.51	1.91	0.60	0.020	+0.002
	184.2	163.2	21.0	24.9	2.45	1.88	0.57	0.019	+0.001
	185.2	163.7	21.5	24.3	2.51	1.91	0.60	0.020	+0.002
	184.0	163.7	20.3	24.8	2.37	1.88	0.49	0.016	-0.002
	180.4	157.3	23.1	18.9	2.78	2.19	0.59	0.019	+0.001
50 cc $S = 3.5$ cc $a = 0.500$ cc 1 cc samples of Na_2CO_4 solution. Concentration = 40.00 mm per liter including CO_2 content of water.									
L	316.0	204.5	111.5	24.0	3.33	2.56	0.77	0.019	+0.001
	314.2	205.0	109.2	23.7	3.27	2.58	0.69	0.017	-0.001
	301.5	192.2	109.3	24.0	3.27	2.56	0.71	0.018	0.000
	298.0	190.5	107.5	23.5	3.22	2.59	0.63	0.016	-0.002
Average								0.018	± 0.0013

results are given in Table VIII. They indicate an average reabsorption equal to 0.018 of the CO_2 in the analyzed sample of solution, hence an α factor of 1.018. The latter confirms within the limit of error the value 1.017 obtained from the results in Tables III and IV.

TABLE IX

Factors by Which Millimeters P_{CO_2} are Multiplied to Give Milligrams CO_2 in the Sample Analyzed. 50 Cc. Apparatus.

Temperature °C	S = 20 cc.		S = 3.5 cc		S = 70 cc	
	a = 0.5 cc t = 1.037	a = 2.0 cc t = 1.017	a = 0.5 cc t = 1.037	a = 0.5 cc t = 1.017	a = 0.5 cc t = 1.037	a = 2.0 cc t = 1.017
10	0 001366	0 005357	0 001419	0 005570	0 001560	0 006121
11	58	29	11	34	48	6068
12	52	03	01	01	34	17
13	46	5278	1394	5469	22	5969
14	38	50	86	36	10	22
15	32	26	78	06	1498	5878
16	26	00	70	5374	86	31
17	20	5175	62	45	75	5787
18	12	51	56	17	65	44
19	07	27	48	5287	55	04
20	01	06	40	62	45	5665
21	1297	5084	34	34	35	29
22	91	60	26	06	25	5590
23	85	36	20	5179	15	50
24	79	15	14	53	05	15
25	73	4993	07	27	1397	5479
26	67	71	01	02	88	42
27	63	51	1295	5078	80	08
28	57	30	89	54	70	5376
29	51	10	83	30	62	44
30	47	4890	77	07	54	13
31	41	70	71	4985	48	5285
32	37	51	65	63	40	56
33	31	31	59	42	32	26
34	27	13	53	20	26	5198

Computation of Tables of Factors for Use in CO_2 Determinations

With the ι factors above determined, total factors for use in analyses covering the ordinary range of conditions were computed by Equations 4, 5, and 6. The factors are given in Tables IX to XI. Their exactness has already been demonstrated by the analyses on which they are based in Tables III to VI.

TABLE X

Factors by Which Millimeters P_{CO_2} are Multiplied to Give mm CO_2 per Liter in Solution Analyzed. 50 Cc Apparatus

Temperature °C	Sample = 0.2 cc	Sample = 1.0 cc					
	$S = 2.0$ cc $a = 0.5$ $z = 1.037$	$S = 2.0$ cc		$S = 3.5$ cc		$S = 7.0$ cc	
		$a = 0.5$ cc $z = 1.037$	$a = 2.0$ cc $z = 1.017$	$a = 0.5$ cc $z = 1.037$	$a = 2.0$ cc $z = 1.017$	$a = 0.5$ cc $z = 1.037$	$a = 2.0$ cc $z = 1.017$
10	0 1552	0 0310	0 1218	0 0323	0 1266	0 0355	0 1391
11	44	09	11	21	58	52	79
12	36	07	05	19	50	49	67
13	29	06	0 1199	17	43	46	57
14	21	04	93	15	36	43	46
15	14	03	88	13	29	41	35
16	07	01	82	11	22	38	25
17	0 1499	00	76	10	15	35	15
18	92	0 0298	71	08	08	33	06
19	86	97	66	06	02	31	0 1297
20	79	96	60	05	0 1196	28	88
21	72	94	55	03	90	26	79
22	66	93	50	02	83	24	70
23	59	92	45	00	77	22	62
24	53	91	40	0 0299	71	19	53
25	43	89	35	97	65	17	45
26	40	88	30	96	60	15	37
27	34	87	25	94	54	13	29
28	28	86	20	93	49	11	22
29	22	84	16	91	43	10	15
30	16	83	11	90	38	08	08
31	11	82	07	89	33	06	01
32	05	81	02	88	28	05	0 1195
33	00	80	0 1098	86	23	03	88
34	0 1394	79	94	85	18	01	82

To obtain mm per liter factors for a sample other than 1 cc, divide the above factors for 1 cc by the cc of sample analyzed *e g*, for a 2 cc sample, *S*, *A*, and *a* being the same, the factors are one-half of those for a 1 cc sample

To calculate milligram molecules of CO_2 in the actual portion of solution analyzed, use the above mm per liter factor for 1 cc sample, divided by 1000

TABLE XI

Factors by Which Millimeters P_{CO_2} are Multiplied to Give Volumes Per Cent CO_2 in Solution Analyzed 50 Cc Apparatus

Temperature	Sample = 0 2 cc	Sample = 1 0 cc					
	$S = 2 0$ cc $a = 0 5$ cc $z = 1 037$	$S = 2 0$ cc		$S = 3 5$ cc		$S = 7 0$ cc	
		$a = 0 5$ cc. $z = 1 037$	$a = 2 0$ cc $z = 1 017$	$a = 0 5$ cc $z = 1 037$	$a = 2 0$ cc $z = 1 017$	$a = 0 5$ cc $z = 1 037$	$a = 2 0$ cc $z = 1 017$
°C.							
10	0 3454	0 0691	0 2710	0 0718	0 2818	0 0789	0 3097
11	37	87	0 2696	14	00	83	70
12	19	84	83	09	0 2783	76	44
13	03	81	70	05	67	70	20
14	0 3386	77	56	01	50	64	0 2996
15	70	74	44	0 0697	35	58	74
16	54	71	31	93	19	52	50
17	38	68	18	89	04	46	28
18	22	64	06	86	0 2690	41	06
19	07	61	0 2594	82	75	36	0 2886
20	0 3292	58	83	78	62	31	66
21	78	56	72	75	48	26	48
22	63	53	60	71	34	21	28
23	48	50	48	68	20	16	08
24	34	47	37	65	07	11	0 2790
25	20	44	26	61	0 2594	07	72
26	06	41	15	58	81	02	53
27	0 3193	39	05	55	69	0 0698	36
28	79	36	0 2494	52	57	93	20
29	66	33	84	49	45	89	04
30	53	31	74	46	33	85	0 2688
31	40	28	64	43	22	82	74
32	28	26	54	40	11	78	59
33	15	23	44	37	00	74	44
34	03	21	35	34	0 2489	71	30

To obtain factor for a sample other than 1 cc, divide the above factors for 1 cc by the cc of sample analyzed *e g*, for a 2 cc sample the factors are one-half of those for 1 cc

To calculate cc of CO_2 , measured at 0°, 760 mm, in the actual portion of solution analyzed, use the above volume per cent factors for 1 cc samples divided by 100.

The same i values have been used to calculate the factors for analyses with different S values, since it has been shown by Van Slyke and Stadie that under the conditions of the analyses re-absorbed CO_2 is a constant proportion of the amount of CO_2 present, and is independent of the volume of water solution on the surface of the mercury, when the mercury is completely covered

When the 100 cc apparatus is used, the factors for the 50 cc. apparatus may be applied provided the relationships, *sample: a S : A* are the same. *Eg*, for analysis of a 2 cc. sample in the 100 cc apparatus with $S = 7$ cc and $a = 4$ cc., one uses the same factors in calculating volumes per cent CO_2 as in analysis of a 1 cc sample in the 50 cc apparatus, with $S = 3.5$ cc and $a = 2$ cc. For conditions other than those defined by the tables, one may readily calculate proper factors by means of Equation 4, 5, or 6. The values for α' and $\frac{1}{1 + 0.00384 t}$ required for calculating such a table of factors are given in Table I of Van Slyke and Neill's paper, and the manner of simplifying the calculations by combining constants is described at the bottom of pp 541 and 542 of the same paper

SUMMARY.

The factors for calculating the results of CO_2 analyses in the manometric gas apparatus have been submitted to rigid experimental tests with standard Na_2CO_3 solution, the accuracy of which was controlled to approximately 1 part per 1000 by three independent modes of analysis, gravimetric Na and CO_2 determinations, and alkalimetric titrations

On the basis of results obtained with these standard solutions tables of analytical factors have been computed with increased precision.

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THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

VII. THE EFFECT OF IRRADIATED CHOLESTEROL ON THE PHOSPHORUS AND CALCIUM BALANCE.

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For the past 3 years experiments have been carried out in this laboratory on the effect of irradiated substances on the bony structure of various animals, more particularly of rats. During the latter part of this period irradiated cholesterol or phytosterol has been fed rather than the crude substance itself. It was shown that where a deficiency of either phosphorus or calcium existed in the blood—a manifestation which is characteristic of rickets or of tetany—this defect could be promptly and regularly alleviated either by adding irradiated cholesterol to the dietary or by supplying it by the subcutaneous route. This result was accomplished in children, in dogs, and in rats. But, as stated, by far the greatest number of tests was carried out on rats. Although this specific action of irradiated cholesterol has been demonstrated by means of chemical analyses of the blood, radiographs of the bones, and histologic examinations, no metabolism tests have been carried out. It seemed worth while, therefore, to undertake an investigation of this nature.

Some experiments were carried out on dogs. Irradiated cholesterol was fed in large quantities to a series of normal dogs, a gm. being given every 24 hours in order to ascertain whether by this means the calcium content of the blood could be raised. It was found regularly, however, that the calcium could not be increased above the normal, in other words above 10.0 to 11.0 mg. per 100 cc of serum. As it is extremely difficult to raise the calcium of the blood above its normal level, experiments were next undertaken to

TABLE I

Effect of Feeding Irradiated Cholesterol to a Parathyroidectomized Dog.

Hrs after operation	Irradiated cholesterol	Serum calcium	Food	Calcium lactate	Clinical signs, etc
	gm	mg per 100 cc		gm	
0		14.5	Irradiated cholesterol (2 gm.).		Hound ♀, 16 kilos.
4	1.0	11.8	Meat 15 gm		Blood taken just before parathyroidectomy
24	1.0	8.2	" 15 "		Active No tetany
48	1.0	7.33	Milk 25 cc.		Left hind leg very stiff No other symptoms.
52		7.8			Panting No tremors Legs stiff. Refused meat
74	1.0		Meat 15 gm Dry milk 15 gm (in 50 cc. H ₂ O)		Lying down. Sleepy
78		6.3			Slight tremors (?)
92	1.0		Dry milk 15 gm		Very sluggish, but walks normally
96					Panting slightly. Tremors
98	1.0	5.4	Milk 200 cc		Convulsion
108			" 80 "	20	Walks a few steps and falls
116	1.0				No improvement
119		6.7	Milk 150 cc (by tube)	8	Condition very poor Spasmodic jerking followed by periods of exhaustion.
129	1.0		Bread 25 gm Milk 400 cc		Lying down No tremors
140			Bread 30 gm Milk 500 cc		Walks without falling
167	1.0	4.0	Milk 300 cc		Lying down No tetany
174			" 150 "	15	Tremors Walks stiffly
190		3.8			No tremors Lying down
					Dead Bled at once from heart

ascertain whether the calcium of parathyroidectomized dogs could be maintained at a normal level by giving irradiated cholesterol. As all these experiments resulted in failure, it seems unnecessary to record them in detail. A typical example is reproduced in Table I. It will be seen that in spite of the fact that 1 gm of irradiated cholesterol was fed daily to this dog, the calcium fell steadily, reaching the level of 3.8 mg. just preceding death. Signs of tetany also developed, in spite of the fact that the diet of the dog included liberal amounts of milk

Subsequent investigations were carried out on rats. Male animals were chosen, 3 to 4 weeks old, and kept on the experimental ration 5 days before being put in the metabolism cage. The cage, which held two rats, was of the type manufactured by the Wahmann Manufacturing Company. Its chief advantage is that it has a floor which can be removed easily for cleaning, and a food cup which can be detached and refilled without disturbing the animals. Collections were made only once during the metabolic period. It was found that no precautions were sufficient to prevent entirely the contamination of the urine with traces of food and with dust from the bodies of the animals. Since the error introduced in this way was too great to be neglected except in a large series of experiments, determinations were made on urine combined with cage washings and the uneaten food residue. Analyses were done in duplicate; calcium was determined by McCrudden's method, total phosphorus volumetrically, following Hibbard's modification. In the first series normal animals were used and were given a dietary which was adequate although not perfectly balanced. It consisted of the standard No. 84 low phosphorus diet containing 10 per cent dry milk and 2 drops of cod liver oil. In other words, it was the standard rachitogenic diet which had been amplified in order to supply its various deficiencies. The rats weighed 70 and 94 gm respectively, were of Wistar Institute stock, and gained well throughout the experimental periods. The data of these two periods, each of which was continued for 7 days, may be seen in Table II. The result may be summarized by the statement that there was moderate retention of both phosphorus and calcium, a balance approximately of 5.2 and 7.6 mg of the former and 5.8 and 9.8 mg of the latter. These tests with normal rats were run rather as a check on experimental conditions, for example

TABLE II.
Phosphorus and Calcium Metabolism of Normal Rats

	Weight	Diet	Experiment 1 (7 day period)				Experiment 2 (7 day period)			
			Phosphorus		Calcium		Phosphorus		Calcium	
			Per capita daily				Per capita daily.			
			gm	gm	gm	gm.	gm	gm	gm	gm.
Gross intake.	70-92	Non-rachitogenic diet, No. 84, low phos- phorus ration with 10 per cent dry milk sub- stituted for equiva- lent amount of flour, plus 2 drops cod liver oil daily.	0 0118	0 0251	0 0432	0 0158	0 0328	0 0580		
Output	94-104		0 0044	0 0123		0 0055	0 0154			
Feces			0 0022			0 0027				
Urine and food			0 0066			0 0082				
Total.			+0.0052		+0.0058	+0.0076		0 0482		
Balance								+0.0098		

on the efficacy of the cage, the completeness of collections, etc., rather than as a control for comparison with subsequent tests in which irradiated cholesterol was fed. The dissimilarity in the diets of the normal and the rachitic rats, with their inherent differences in phosphorus and calcium content would make comparison difficult

In the tests on rachitic rats the standard No. 84 (Sherman-Pappenheimer) ration was used with the addition of 5 per cent dry milk. The substitution of this percentage of dry milk for an equivalent amount of flour compensates to a certain extent for the deficiencies of this diet without, however, affecting the degree of rickets, the rats grow better with the supplement than on the unmodified No. 84 diet. Two series of experiments were carried out with rachitic rats. In the preliminary periods 2.5 mg. of non-irradiated cholesterol dissolved in linseed oil were fed by mouth in addition to the ration which is low in phosphorus and high in calcium. During the experimental periods in both tests 2.5 mg. per capita of irradiated cholesterol were given daily in place of ordinary cholesterol. The preliminary period in the first experiment lasted for 5 days and in the second for 7 days. The test periods, during which irradiated cholesterol was given, were of 5 days duration in the first instance and of 7 days in the second.

The phosphorus intake of the animals is small on this ration, the per capita being 7.0 to 8.0 mg. during each of the 5 day periods and 9.0 to 10.0 mg. during each of the 7 day periods. It could not, therefore, be anticipated that an increase in retention would be of large amount. It will be noted, however, that during the two periods of the first as well as in those of the second experiment, an increased retention of phosphorus was brought about.

In regard to the calcium, the figures are somewhat higher as the intake was decidedly greater. During the 5 day periods of the first experiment the intake of calcium ranged between 35 and 38 mg. and during the 7 day periods of the second experiment the amounts ranged from 45 to 48 mg. Whereas in the preliminary tests, there was almost no positive balance or even a slightly negative balance, it will be noted in Table III that there was a positive balance of 1.8 mg. in the course of each 5 day test, and that during the two 7 day periods the positive balance was 2.6 and 2.8 mg. respectively. The difference brought about in the excretion of

calcium by way of the alimentary tract is shown significantly in the second experiment. In the preliminary period approximately 30 mg. of the 47 mg. consumed in the food were excreted by the bowel. On the addition of irradiated cholesterol to the diet only 12 of 45 mg. of calcium were lost by this path in the first test, and only 13 of the 48 mg. consumed were excreted in the feces in the second test.

Irradiated cholesterol has been found to raise the phosphorus

TABLE III—*Effect of Irradiated Cholesterol on the*

Experiment 1	Weight	Diet	Preliminary 5 days			
			Phosphorus		Calcium.	
			Per capita daily			
	gm		gm	gm	gm	gm
Gross intake	66-70	Rachitogenic diet, No. 84, low phosphorus ration with 5 per cent dry milk substituted for an equivalent amount of flour, plus 2.5 mg non-irradiated cholesterol in linseed oil		0 0081		0 0398
Output	79-92					
Feces			0 0052		0 0296	
Urine and food			0 0025		0 0120	
Total				0 0077		0 0416
Balance			+0.0004		-0.0018	
Experiment 2			Preliminary 7 days			
Gross intake	88-90	Same as in Experiment 1		0 0098		0 0477
Output	90-92					
Feces.			0 0062		0 0308	
Urine and food			0 0030		0 0167	
Total.				0 0092		0 0475
Balance			+0.0006		+0.0002	

and calcium content of the blood in rachitic animals. Numerous tests which we have carried out during the past few years have shown that the inorganic phosphorus of the blood falls from about 8 mg. to 2.5 mg. per cent when rats are placed on the No. 84 diet, even though reinforced by 5 per cent dry milk. When 2.5 mg. of irradiated cholesterol are added to this dietary, the inorganic phosphorus content is raised to about 4.25 mg. per cent. It is difficult to increase this percentage still further by augmenting the amount of irradiated cholesterol. If 25 mg. per capita are fed

daily, the inorganic phosphorus content of the blood is but slightly enhanced. When, however, very large amounts are given, as great as 100 mg. daily, the phosphorus content has reached 5.77 mg. Evidently this diet is too deficient in phosphorus to enable the blood to attain the normal value.

CONCLUSIONS.

These experiments indicate that irradiated cholesterol, when

Phosphorus and Calcium Metabolism of Rachitic Rats

Diet	Period 1 (5 days)				Period 2 (5 days)			
	Phosphorus		Calcium		Phosphorus		Calcium	
	Per capita daily				Per capita daily			
	gm	gm	gm	gm	gm	gm	gm	gm
Rachitogenic diet, No 84, low phosphorus ration with 5 per cent dry milk substituted for an equivalent amount of flour, plus daily 2.5 mg. irradiated cholesterol in linseed oil.		0 0071		0 0349		0 0078		0 0382
	0 0045		0 0226		0 0044		0 0246	
	0 0020		0 0105		0 0026		0 0118	
		0 0065		0 0331		0 0070		0 0364
		+0.0006		+0.0018		+0.0008		+0.0018
	Period 1 (7 days)				Period 2 (7 days)			
Same as in Experiment 1.		0 0093		0 0454		0 0098		0 0481
	0 0057		0 0124		0 0053		0 0130	
	0 0028		0 0065		0 0029		0 0071	
		0 0085		0 0189		0 0082		0 0201
		+0.0008		+0.0265		+0.0016		+0.0280

given by mouth or subcutaneously, is unable to raise the calcium content of the blood above the normal. Nor is it able to prevent the fall of calcium brought about by parathyroidectomy.

Metabolism tests show, however, that irradiated cholesterol when added to a rickets-producing ration which is low in phosphorus, brings about a definite increase in the retention of phosphorus and calcium.

HUMAN MILK STUDIES.

I. TECHNIQUE EMPLOYED IN VITAMIN STUDIES.

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As a part of a series of studies involving the chemical and biological aspects of human milk production these data on the standardization of procedures for estimating quantitatively the vitamin content of breast milk have been compiled. The methods used here have included the newer technique of Steenbock and his collaborators (1-3), Evans and Bishop (4), Sherman and associates (5-7), and others, who have elaborated upon the classic methods of Osborne and Mendel as described by Ferry (8). Using a combination of the above methods, the standards for growth, reproduction, and lactation have been worked out for the *conditions existing in these laboratories*, in respect to breed of rat, character of food, method of feeding, type of cage, etc., factors which may vary somewhat from other laboratories. The experiments have not been conducted with the purpose of determining the "minimum amount of the material under examination which suffices to produce a standard result in or for a standard time," as suggested by Sherman and Munsell (5), but with the purpose of obtaining *optimal physiological behavior*, such as growth, reproduction, and lactation in the rat with an attempt to learn more about ideal nutritional conditions for comparable functions in the human species. In this paper most of the data concerning the deportment of animals on the purified regimens, complete or otherwise, are presented for purposes of comparison with the subsequent work on the quantitative determinations of vitamins in human milk.

Preparation and Standardization of Test Animals.

In view of the fact that the deportment of young rats held on experimental rations depends to a large extent on the character of the diet given the mother during the breeding period, the practice of using only those offspring of rats that have been reared in this laboratory has been followed. Two distinct strains of rats have been used: the albino rat of the Wistar Institute stock, and the pied rat from the McCollum colony¹. Extreme care has been exercised in maintaining conditions that are quiet, peaceful, and hygienic. The young are handled from the day of birth and become very docile and tractable. All stock animals are kept in small individual metal cages, of the type described by Long and Evans (9). Sterilized shavings, used for bedding, are changed three times a week, while the trays are washed and *sterilized* once every week, thus insuring clean, hygienic conditions for the rearing of young.

The diet formulated by Sherman (10), consisting of two-thirds whole wheat, one-third whole milk powder,² and NaCl to the amount of 2 per cent of the wheat, has been used as the stock ration, in addition, fresh cabbage or lettuce has been given 6 days a week. The ration of the lactating rat has been further augmented by fresh cow's milk *ad libitum*.

Special care has been given to the selection of the best animals for the propagation of the colony and production of superior young for experimental purposes, with the view of standardizing as many factors as possible. Although the females have become sexually mature between the 35th and 42nd day of life, with ovulation following approximately every 4th to 5th day thereafter, breeding has not been carried out until after the 4th or 5th month. The gestation period, counting the day that sperm are found in the vaginal tract as the first day, until the litter is cast, is 23 days in length. On the day of parturition the litter is weighed and reduced to six in number. The maternal rat, when in good health and nursing six offspring for a period of 3 weeks,

¹ The authors desire here to express their gratitude to Prof. E. V. McCollum, of Johns Hopkins University, who kindly furnished the original stock rats.

² Klim, a powdered whole milk product secured from the Merrell-Soule Co., Syracuse, N. Y.

is able to gain in weight an average of 15 gm. with a standard deviation of ± 10 . The young are weaned on the 21st day of life and the mothers are then allowed a recuperative period of at least 2 months before being bred again. In Table I is shown the mean growth of the young each week during the nursing period, and the deviation from the average which may be expected in so called normal young

Following the suggestion of Robertson and Ray (11) a biometric interpretation of the growth of all males and females reared on

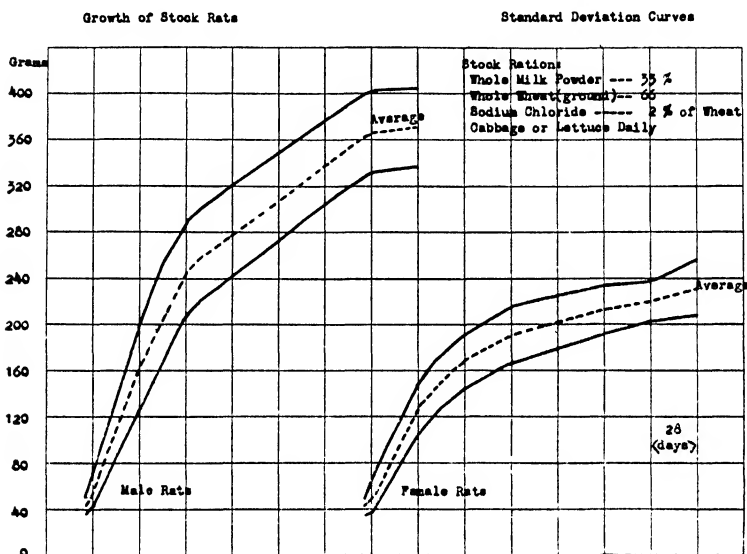


CHART I Standard deviation curves of male and female rats reared to maturity on the stock ration

the stock ration has been made, and is recorded graphically on Chart I. The middle lines (dotted) show the mean growth curves of the groups. The solid lines represent the maximum and minimum variation of the animals at different ages as determined by estimating the standard deviation for the series. In plotting the curves the total area obtained is twice the standard deviation, and represents the limits of normal variability of the animals. All growth curves falling within this area, consequently, may

justly be considered normal; those falling below the lower limit have been arbitrarily considered subnormal. This includes the one-sixth of the total number of curves in the series regardless of the fact that the animals were apparently normal and receiving an adequate ration. Concerning the other one-sixth of the curves which fall above the maximum limit there exists a difference of opinion. Robertson and Ray (11) have taken the view that these rats are not normal. In this laboratory with the type of studies under investigation, animals exhibiting this superb growth have been considered normal, especially since it has been found that young rats weighing 50 gm. at 21 days of age have not been able to store in their tissues more of vitamins A and B than have rats of the same age weighing only 36 to 40 gm. McCollum and Simmonds (12) state, moreover, that "it would be more logical to accept only the greatest gain observed in any animal, rather than to average the gains made by good and poor ones "

This difference in the rate of growth and in the adult maintenance weight of rats exhibiting no obvious macroscopic signs of disease, has been recognized by the majority of investigators who have used the rat in carefully controlled experiments. Among various explanations for this phenomenon is the possibility of the existence of foci of infections or pathological lesions not attributable to diet. Givens and Behrendt (13) have stated that the variability in growth may be due to a difference in the amount of food eaten by individual animals. Osborne and Mendel (14, 15) have shown that there is a progressive improvement in the rate of growth of animals in their colony from year to year, a condition which has been marked in this laboratory during the 2 years of its existence. The use, therefore, of a monotonous unchanging well balanced breeding ration together with the method of breeding vigorous females with the same degree of frequency, the reduction of all litters to six in number, and the practice of placing only litter mates on a given ration whenever possible have been instituted in order to reduce to a minimum this great variability in the deportment of rats on identical rations

EXPERIMENTAL.

In the experimental investigations in this laboratory only young vigorous animals in excellent condition have been employed. Those weighing less than 40 gm. on the 21st day of life have seldom been used, although the range of normal growth as determined by the standard deviation from the average includes rats between the weights 36 and 50 gm. (see Table I). The age at which the animals are put on experimental rations may vary from 21 to 26 days, depending on the type of investigation. Each rat is confined to a standard individual cage, the tray of which is covered with a sterilized heavy absorbent paper³ held down by a $\frac{1}{2}$ inch mesh screen. This type of bedding allows not only for recovery of any food scratched out of the food cup, but also for observation on the character and quantity of feces excreted. Distilled water, clean and fresh, is available at all times.

TABLE I

Growth of Offspring of Female Rats Reared on a Natural Food Regimen.

Age	Average weight per rat	Standard deviation
<i>days</i>	<i>gm</i>	<i>gm</i>
7	14	± 3
14	30	± 5
21	43	± 7

Basal Ration.

The standard basal ration is practically fat-free. Its components are:

	<i>per cent</i>
Casein, purified ⁴	18
Dextrin	76

³ A bleached soda fiber, made of poplar wood by the alkali process, and bleached with chlorine by the Penobscot Chemical Fibre Co., 211 Congress Street, Boston.

⁴ The procedure for the purification of the dry pulverized crude casein includes washing with acidulated distilled water for 7 days according to the McCollum and Davis method (17), subsequent drying at a temperature below 100°C., and pulverizing. This is followed by treatment with alcohol, using essentially the procedure used by Sherman and Munsell (5) with the

Salt mixture (16) .	4
Agar agar ⁵ .	2

The casein⁶ is thoroughly extracted with water, alcohol, and ether, a process which renders it essentially pure and vitamin-free, as demonstrated by feeding experiments. The same casein is used in both the vitamin A and B experiments, in order that all results may be comparable. Starch⁷ is partially dextrinized by pouring moistened starch into boiling water, cooking until the mass becomes clear and opalescent, and drying in a current of air at 120°C. This procedure is carried out to increase the palatability of the food and to facilitate digestion, which aid may be of importance in the vitamin A-free rations if the secretory cells of the salivary glands undergo marked pathological changes as reported by Mori (18) and Wolbach and Howe (19). A dry fat-free basal ration has been used throughout, because of its simplicity and ease in preparation and feeding, its stability on exposure to light, high temperature, and long standing.

Standard Complete Ration.

In order to make the basal ration complete and to satisfy all the requirements for normal physiological function in the rat, small quantities of vitamin-carrying substances are fed in small glass dishes apart from the basal ration. These adjuvants are given six times per week, with a double quantity on Saturday, in the following proportions: 5 drops (100 to 120 mg.) of a stand-

exception that 80 per cent alcohol is used for the first extraction, in order to allow for more thorough lixivation of the casein granule with 95 per cent alcohol. The casein is then dried, pulverized, and extracted continuously for 48 hours with U S P. ether. It has been demonstrated by feeding experiments that the extraction of the crude casein with acidified water over a period of 7 days was sufficient to remove practically all of the vitamin B. The subsequent treatment with alcohol and ether has been carried out to remove more of the fat-soluble substances, such as vitamins, pigments, fats, etc., in order to secure a more highly purified casein.

⁵ Granular agar-agar obtained from the Arthur H. Thomas Co., West Washington Square, Philadelphia

⁶ Casein made by the California Central Creameries, San Francisco, was used in these experiments.

⁷ Argo Corn Starch made by Corn Products Refining Co., New York, was purchased at the local markets.

ardized cod liver oil⁸ supply vitamin A and the antirachitic factor; 2 drops (40 to 50 mg.) of an anhydrous ether extract of dried wheat germ⁹ add the antisterility vitamin; 0.4 gm. of yeast,¹⁰ accurately weighed, furnishes vitamin B as well as other possible substances described by Funk and Dubin (20) and Goldberger

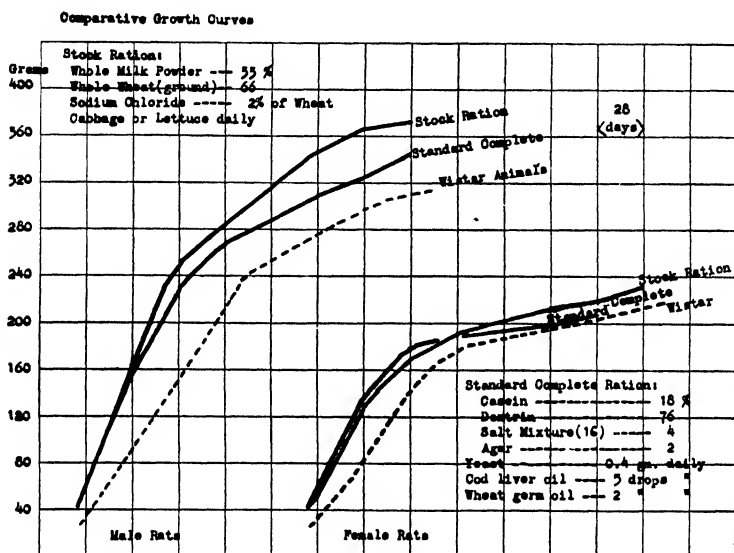


CHART II Average growth curves of all male and female rats held on the stock ration, the standard complete ration, and the carefully selected group from the Wistar Institute

and Lillie (21). Of the different varieties^{11,12} of yeast used in extensive experiments with rats on *false screens*, that obtained

⁸ Cod liver oil No 1610, Mead Johnson Company, Evansville, Ind.

⁹ Gold Medal wheat germ, Washburn Crosby Company, Minneapolis.

¹⁰ The authors appreciate the courtesy of Dr M H Givens in securing this product from the Northwestern Yeast Company, 1750 North Ashland Avenue, Chicago

¹¹ We are indebted to Dr. Robert E. Lee, of the Fleischmann Yeast Company, 699 Washington Street, New York, who kindly furnished yeast for some of these experiments.

¹² Vitamin-Harris Concentrates 1001 and 1003 and yeast, dried powdered, tested for vitamin B activity, obtained from the Harris Laboratories, Tuckahoe, N. Y.

from the Northwestern Yeast Company has been selected to furnish vitamin B in the more recent experiments, since 0.4 gm. of this product, when fed to rats, has produced continuous growth at a rate equivalent to that of animals on a natural food regimen. Chart II illustrates the success of this standard complete ration. In appearance the rats are sleek and clean with fine glossy hair; their bodies feel firm and they are very active. On a level of 0.3

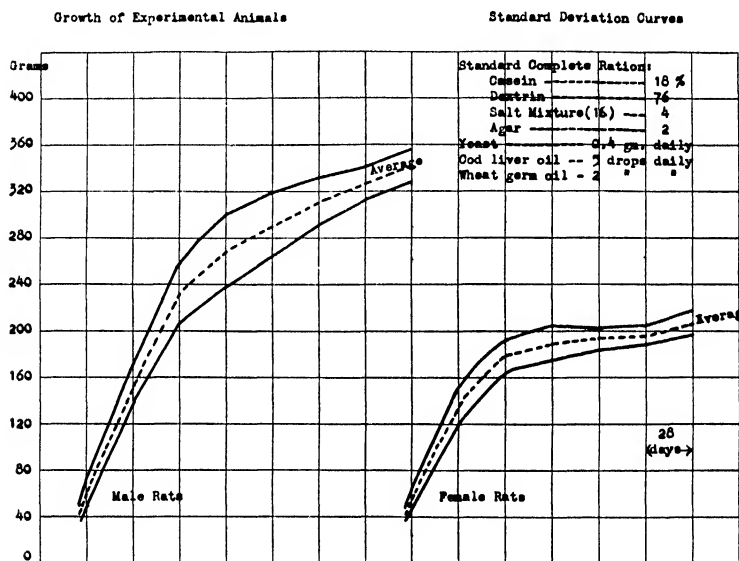


CHART III Average growth and standard deviation of males and females reared from 21 days to maturity on the standard complete ration

gm. of Northwestern yeast daily, average but not optimal growth has been obtained.

All animals are weighed either once or twice each week between 8 and 10 o'clock in the morning before the daily feeding. The basal ration is fed *ad libitum*, and accurate records of the food consumed weekly by individual animals are kept. Examination of the vaginal epithelium of the females is made daily.

On Chart II is recorded the average growth of both the males and females receiving the purified basal ration plus the daily vitamin additions. For purposes of comparison there are included curves showing the average growth of the stock animals in this

laboratory and also a curve constructed from the growth data of a selected group of the Wistar Institute rats (22). By the 112th day the growth curve representing the average of the experimental males diverges definitely from the curve exhibited by the stock males. It is, however, above the minimum normal limit as defined by the standard deviation from the average of the stock rats.

Chart III demonstrates graphically the standard deviation from the average of the group of males and females receiving the standard complete ration. It is noticeable that, although the adult males do not grow quite as well as the stock adult males, there is less variation in their deportment, as demonstrated by the markedly narrower normal area defined by the maximum and minimum standard deviation curves. It is of interest to note, too, that in this laboratory there is less variability among the females receiving either the stock or the purified diets than among the males on identical rations. In view of the fact that the deportment of the animals receiving the standard complete ration so nearly approximates that of animals held on a natural food regimen, their growth has been considered normal. It is, therefore, with this control group that the physiological behavior of all the animals on other experimental rations is compared.

The females receiving this standard complete ration have become sexually mature about the 42nd day of life, and have ovulated every 4 to 5 days. The time of opening of the vaginal orifice has been found to be apparently dependent on the amount of vitamin B in the ration. Evans and Bishop (4) have shown that "the study of the ovulation rhythm furnishes a new, a different and a more sensitive test of physiological well-being than that furnished by growth rate." In this laboratory a definite retardation in sexual maturation as defined by the opening of the vaginal orifice (23) and in the establishment of normal rhythmical estrous cycles has been encountered many times when yeasts of low vitamin B potency have been used, although fairly good growth was obtained. Throughout the experiments, then, the age at sexual maturity, the length of the estrous cycles, and the character of the vaginal epithelium, as well as the rate of growth, have been used as criteria of an adequate ration.

Reproduction and Lactation.

In studying reproduction and lactation the motive has been particularly to determine the *adequacy* of the standard complete ration for reproducing and rearing young, and to establish a definite standard with which to compare results from subsequent diets including milk. For this investigation, females reared from the 21st day of life to maturity on the standard complete ration were used. They were bred with fertile stock males at estrus about the 120th day of life. All conditions were maintained the same as during the growth period, including the use of false screens. Reproduction has regularly been secured on the basal ration containing the same amount of the vitamin-bearing substances as were essential for optimal growth. When 2 drops of the wheat germ extract have been fed daily from the 21st day of life throughout the experiment, no resorptions have been noted. Litters, the size and appearance of those secured on the stock diet, were always found. During pregnancy no consistent increment in food consumption has been noted. These animals eat normally from 60 to 80 gm. of the basal ration weekly. To facilitate the comparison of these data with those of other investigators, the amount of yeast used has been calculated to be approximately equivalent to 3.5 to 4.5 per cent of the ration while the wheat germ oil represents about 0.4 to 0.6 per cent of the ration. There is a diversity of opinion concerning the necessity of vitamin E in the diet, principally because of its general distribution in foods, its resistance to heat and desiccation, and its storage in the animal body. Evans and Burr (24) have shown conclusively its rôle and have secured fertility when 25 mg. of wheat germ oil were fed daily from the 23rd day of life.

Lactation experiments have been conducted with the view of determining: first, the ability of the rat to rear successfully litters of six young on the standard complete ration; second, the amount of yeast and wheat germ oil required for optimal growth of the young and prevention of loss of weight in the mother; third, the food requirement of the lactating mother; and fourth, the necessity of milk in the diet of the lactating rat as reported by Suguira and Benedict (25). In all experiments the amount of cod liver oil and the percentage of components in the basal ration have been the same as that used for growth and reproduction.

TABLE II
Growth of Litters and Food Intake of Rats Receiving Different Amounts of Yeast and Wheat Germ Oil

Rat No	Daily amounts of			No of young found *	Weight of young			No of young weaned	Weight of mother, loss or gain.	Basal food intake of mother			Per cent of ration		Remarks
	Yeast	Wheat germ oil	Cod liver oil		7th day	14th day	21st day			1st wk	2nd wk	3rd wk	Yeast	Wheat germ oil	
416	3 0	10	5	9	17	30	44	6	-18	132	145	184	13 6	1 1	Ration containing 22 per cent fat Own young eaten Given 4 young from stock litters.
659	3 0	4	5	9	14	26	39	6	+13	105	190	213	12 4	0 41	
238	2 0	15	5	10	10	23	40	5	+35	123	152	209	18 7	1 6	
329	2 0	10	5	4	12	25	43	4	+4	95	115	147	11 7	1 4	All but 1 eaten Given 4 young from stock litters.
331	2 0	8	5	6	12	23	37	6	+9	63	119	126	13 7	1 4	
315	2 0	8	5	5	13	27	43	5	+22	67	148	150	11 5	1 6	
313	2 0	8	5	9	14	27	40	6	+2	111	192	214	8 0	0 8	Ration containing 22 per cent fat
417	2 0	8	5	8	11	24	39	5	-7	100	144	181	9 9	0 9	
353	2 0	8	5	5	13	25	39	4	-8	101	132	140	11 2	1 1	
350	2 0	8	5	8	11	21	35	6	-20	43	87	119	16 7	1 6	
530	2 0	5	5	8	13	24	38	6	-5	104	140	226	8 9	0 5	
530	2 0	2	5	8	14	26	39	6	-12	106	187	232	8 0	0 2	
317	1 5	10	5	10	12	24	40	3	+19	55	98	168	10 1	1 7	
366	1 5	10	5	6	13	27	41	4	+2	76	124	127	9 6	1 6	
531	1 5	10	5	5	14	26	39	5	-23	98	129	189	7 6	1 2	
353	1 5	10	5	5	16	27	39	5	-24	119	127	185	7 3	1 2	
529	1 0	10	5	7	16	28	40	6	-21	119	180	217	4 0	1 0	
656	1 0	10	5	9	13	25	37	5	-6	105	148	199	4 6	1 1	

* All litters reduced to six on 1st day

The only variations made were in the actual amounts of yeast and wheat germ oil given daily. Previous experiments had demonstrated that rats required unusually large amounts of yeast while nursing young. This is in accord with the work of other investigators (26-29).

Female rats, kept in the individual metal cages on false screens, were given sterilized shredded crepe paper¹³ on which to cast their young. Raised screens were used in order to obtain an accurate estimate of the amount of vitamin B required and the basal ration eaten. On the day of parturition the number of rats in each litter was reduced to six, and the amount of wheat germ oil and of yeast was increased in varying proportions for different rats (Table II). Occasionally during the first few days a mother would devour one or two of her new born, which undoubtedly augmented her diet at least in respect to vitamin E, as shown by Evans and Burr (24). Table II also shows the growth of the young of mothers receiving different levels of yeast and wheat germ oil. This table has been included principally for purposes of comparison with the growth of litters on milk experiments. No attempt has been made to compare the growth of litters of less than six young in these experiments, although from an analysis of the breeding records of stock females, it was found that litters of five young have not grown better than those containing the standard number of six. The data on hand are too limited to draw conclusions from, other than that the vitamin B requirements during lactation are increased above normal and that yeast must be fed 3 to 5 times the usual amount if the offspring are to weigh 37 to 50 gm. on the 21st day of life. The demand for increased vitamin E is not as clearly defined. The young successfully weaned on the basal ration plus the daily vitamin additions have all the characteristics of normal vigorous young. When they in turn are placed on the standard complete ration, their growth is as satisfactory as the growth of the stock young on a similar diet. It is apparent then, that lactation is *normal* on synthetic diets, and that when subnormal growth is obtained it is not because of the unsuitability of the standard complete ration but

¹³ White shredded crepe paper purchased from Dennison Paper Company, Framingham, Mass.

because of its inadequacy in respect to its vitamin content, especially vitamin B.

From Table II it is seen that the amount of basal food eaten is increased progressively throughout lactation as has been observed by Suguira and Benedict (25), John and Schick (30), Wang (31), etc. During the 3rd week the average amount consumed per rat was at least twice as great as that during growth and pregnancy. Whether this is a demand for calories or for protein has not yet been determined. The fluctuation in the amount of food consumed has made it impossible to maintain a constant relationship between the yeast and wheat germ oil, and the dry basal ration, for as the actual amount of basal food eaten was increased, the percentage of yeast and wheat germ oil decreased. Table II includes these substances in terms of the estimated percentage of the total basal food consumed during the 3 weeks of lactation. The percentage varies with the individual rats, but in all cases the per cent of wheat germ oil is seen to be lower than that reported by Sure (26) as being necessary for successful lactation.

Rations high in fat containing 2 per cent cod liver oil, 5 per cent butter fat, and 15 per cent Crisco plus 18 per cent casein, 4 per cent salt mixture (16), and 56 per cent dextrin have had no advantage over the comparatively fat-free standard complete ration in respect to growth, reproduction, and lactation. Rats 329 and 330 in Table II give a history of normal reproduction when yeast is increased to 2.0 gm daily and wheat germ oil to 8 to 10 drops daily. These two cases are presented to show that lactation does take place when vitamins B and E are fed in adequate amounts, although Nelson, Jones, and coworkers (28) have reported that rats do not reproduce on high lard diets, even though these diets contain a sufficiency of vitamin A for normal growth and normal reproduction in the form of butter fat.

Vitamin A-Free Ration.

In preparing test animals for vitamin A studies, the curative methods of Steenbock (2, 3) and Sherman (5) have been used. Young rats 21 to 26 days old, reared on the stock ration and weighing a minimum of 38 gm. on the 21st day of life, have been selected for these studies. The basal ration was exposed to the rays of a

mercury vapor lamp¹⁴ at a distance of 15 inches for 30 minutes to endow it with antirachitic properties. Yeast was fed in the usual allotment of 0.4 gm daily, but no cod liver oil or wheat germ oil was given. Since the presence of 2 drops of non-aerated wheat germ oil has deferred the onset of xerophthalmia for 1 to 3 weeks, and since vitamin E is apparently not essential for growth, it has been withheld in the vitamin A studies. On this ration the rats have grown normally for the first 4 to 5 weeks. The growth curves present a very striking picture of the rapidity and uniformity in vitamin A depletion. The animals have developed xerophthalmia about the 34th day of the experiment, irrespective of whether or not they were placed on the vitamin A-free ration on the 21st or the 25th day of life.

TABLE III
Time Required to Produce Vitamin A-Deficiency Symptoms in Rats

Age of normal females at sexual maturity	Appearance of cornified cells in vaginal tract		Appearance of definite xerophthalmia (males and females)		Maintenance or loss in weight (males and females)	
	Age	Days on ration	Age	Days on ration	Age	Days on ration
<i>days</i>	<i>days</i>		<i>days</i>		<i>days</i>	
42±4	46±3	23±3	56±3	34±4	56±3	34±3

For this type of an experiment female rats have been found to be the most satisfactory, since they exhibit greater uniformity in growth and offer opportunity for more extensive physiological observations. Unless adequate vitamin B is given, sexual maturity may be delayed indefinitely, thus losing one of the most delicate tests for vitamin A depletion (32). Daily examination of the vaginal epithelium has shown that although frequently the first estrus may be normal, yet the flaky cornified cells indicative of vitamin A deficiency soon become persistent. Usually, however, this characteristic cell is the only type seen from the time of opening of the vaginal orifice until therapeutic methods have been instituted. In all cases where the vaginal orifice has opened before the onset of xerophthalmia, the presence of the cornified

¹⁴ Alpine Sun Lamp made by the Hanovia Chemical and Manufacturing Co., Newark, N. J.

cells in the epithelial layers of the vagina has been the first and most delicate symptom of vitamin A exhaustion. This has occurred as early as the 23rd day of the experiment (Table III). A few days later a tiny accumulation of exudate in the corner of one or both eyes has been noted, this has been followed in 1 to 3 days by swollen and reddened eyelids and later by extensive exudate. Simultaneously with the appearance of definite xerophthalmia, maintenance or loss in weight has been observed. Concomitant with loss in weight has come a decrease in food consumption. Priapism has been frequently encountered. Another symptom in many of the rats has been the appearance of a disturbance in motor function of the fore legs, suggesting incoordination or partial paralysis, the true significance of which is not yet understood. The condition causes the rats to pivot on their front legs in a manner similar to that of rats with middle ear infections. This disturbance is probably not due to faulty bone formation, since Steenbock and coworkers (33) and Dutcher and Kruger (34) have demonstrated normal bone development in rats fed on rations irradiated with ultra-violet light.

At the end of 4 to 5 weeks on the standard vitamin A-free ration, when xerophthalmia has first been noted, only a few rats have exhibited macroscopic lung lesions. Mastoid cell involvement has not been found with any degree of regularity at this early stage. This is likewise true of abscesses in the salivary glands and glands at the base of the tongue. Lung infections as well as middle ear and mastoid cell involvement have been found, however, when these depleted animals are continued for a week or more on rations slightly or wholly deficient in vitamin A. The retention of rats exhibiting xerophthalmia, loss in weight, etc., indefinitely before curative foods are given should be avoided, since advanced pathological conditions may be established which cannot be ameliorated by vitamin adjuvants (see Table IV). Generalized infections resulting in the localization of pus in the upper part of the digestive and respiratory tracts, *i. e.* tongue and salivary glands, sinuses, mastoid cells, and retropharyngeal areas, apparently cannot be cured by feeding methods, regardless of the richness of the diet in vitamin A. Abscesses still persist even after weeks of excellent feeding, although the growth may be fairly normal. Lung infections become progressively more pronounced; nasal

TABLE IV
Effect of Retention of Rats on Vitamin A-Deficient Rations for Varying Periods and the Ineffectiveness of Therapeutic Feeding after Pathological Lesions Are Established.

Rat No	Time held after appearance of xerophthalmia	Therapeutic feedings		Department of rat after appearance of xerophthalmia	Autopsy findings
		Kind	Days		
708♂	days 0	None.		Disturbance of motor function of fore legs, suggesting incoordination or partial paralysis; died	No macroscopic lesions
709♂	0	"			"
699♂	2	"			"
697♂	8	"		Disturbance of motor function of fore leg Continued severe xerophthalmia	Abscesses at root of tongue, soft palate, and retropharyngeal area.
705♀	8	"		Continued severe xerophthalmia	Liver anemic; pus in both mastoids
703♀	8	"		"	Pus in salivary gland, tongue, and soft palate, and both mastoids. Salivary glands transparent in appearance.
702♀	14	"		"	Pus in tongue, soft palate, and both mastoids Kidneys anemic
				"	Salivary glands transparent in appearance
706♂	10	Cod liver oil, 10 drops daily.	40	Fair growth; lung infection	Congestion of lungs

707 ♀	15	Cod liver oil, 10 drops daily.	33	Subnormal growth	Pus in both mastoids; tiny abscesses at root of tongue
711 ♀	10	"	32	Normal growth	Pus in both mastoids
713 ♀	7	"	32	Nasal hemorrhage; growth curve fluctuating	" " " large abscess at base of tongue (5 mm. diameter)
712 ♀	7	"	11	Normal growth	Lungs anemic, pus in both mastoids. Tongue tissue yellow in appearance
412 ♀		1½ cc human milk daily; then cod liver oil, 5 drops daily	161 70	Subnormal growth containing cod liver oil, weaned one litter subnormal in weight	Pus in both mastoids

hemorrhages occur in some rats. The atrophic condition of the glands secreting digestive fluids, *i.e.* the pancreas and the salivary glands, may account for the inability of a number of animals to respond immediately to vitamin A therapy. The rôle that these abnormalities play in causing fluctuations in weight and a lowered rate of growth is apparent. In view of these facts and the narrow limits of variability among standardized animals in developing symptoms of vitamin A insufficiency, curative feeding should commence before definite xerophthalmia and maintenance or loss in weight are observed, to avoid dealing with grossly pathological animals.

Instead of relying on data obtained from the curative type of experiment alone, additional information may be secured by determining the time required to exhaust the vitamin A depots in offspring of female rats reared on diets containing different amounts of this vitamin. This observation has been made from a study of the relative quantity of vitamin A in the stock ration and in the standard complete ration containing 5 drops of cod liver oil daily. When the young of females reared on these rations were placed, on the 21st day of life, upon the vitamin A-free ration, not only were the growth curves of both groups of rats almost identical, but the characteristic symptoms of vitamin A deficiency occurred after the same number of days. As an average, the cornified cells were observed in the vaginal tract after 24 days, growth ceased by the 33rd day, and definite xerophthalmia appeared on the 34th day, which is within the limits of variability of the standard test animals.

While observations on growth and on changes in the eye tissues and the vaginal epithelium of test animals are valuable criteria in vitamin A studies, yet the continuous study of their offspring furnishes even more significant data. The method of studying the depletion time of the storage of vitamin A in the young of mothers held on special diets can be used very advantageously in obtaining quantitative data on the vitamin A content of food materials.

Vitamin B-Free Ration.

In the vitamin B experiments all animals are kept on false screens without apparent ill effects. Young rats, 21 to 26 days

old, are preferable, although age is not an important factor in these experiments, since there is a question of whether or not vitamin B is stored in any appreciable quantity in the body tissues (35). On the basal ration plus 2 drops of wheat germ oil and 5 drops of cod liver oil daily, young rats have continued to grow for approximately 1 week; this has been followed by maintenance in weight for 1 week or by loss in weight. By the end of the 3rd week the rats have declined markedly in weight; they have become very thin and emaciated; priapism has been characteristic; food consumption has been reduced to a minimum. Death has occurred before the 4th week. Because of the extreme deficiency of the ration in vitamin B, and the intervention of death, very few animals have exhibited advanced polyneuritis.

Consideration of Another Vitamin in Yeast.

The studies of Goldberger and Lillie (21) on the presence in yeast of a thermostable substance, which in itself does not prevent beriberi or allow for normal growth, but which in conjunction with an 85 per cent alcoholic extract of corn does establish good growth, have been repeated in these laboratories. Yeast, heated at 15 pounds pressure for 3 hours, and fed to young rats at a level of 0.4 gm daily, in conjunction with the basal ration plus cod liver oil, has produced growth for 2 to 4 weeks with subsequent decline and polyneuritis within 4 to 6 weeks. All of the animals held on this regimen have exhibited polyneuritis of marked intensity: extreme emaciation, complete loss of equilibrium, spasticity frequently resulting in the animals curling up into a ball, and finally paralysis. This condition has been prolonged in individual rats for as long as a week. The extreme paralysis and length of time between its onset and death are the two features which distinguish it from total yeast deficiency.

When young animals are given the basal ration plus cod liver oil and an 85 per cent alcoholic extract of corn, equivalent to 2 to 4 gm daily, growth has followed for 2 weeks after which there has been maintenance of or slight increase in weight for as long as 28 weeks. The bodies of the animals are long and emaciated. Although a few animals have been held on this ration as long as 10 weeks, no lesions of the eyes, tongue, or skin, described by Goldberger and Lillie as indicative of a pellagra-like condition in rats, have been noted.

Although the inclusion in the ration of either autoclaved yeast or an 85 per cent alcoholic extract of corn has been followed by characteristic conditions as shown in Chart IV, yet the substitution of these two preparations together for the fresh dried yeast in the standard complete ration has resulted in continuous growth. These experiments, as well as those of other investigators (21, 36, 37), would indicate the presence of two factors in yeast. Such facts, therefore, should be taken into consideration in the so called vitamin B studies.

SUMMARY.

1. Using rats as experimental animals, a standardized technique for the quantitative estimation of the vitamin content of human milk is presented.

2. A purified experimental ration has been adopted which produces in rats growth and physiological behavior equivalent to that secured on an adequate natural food regimen. A biometric interpretation of data obtained on both rations has been made

3. Reproduction has regularly been secured on the standard complete ration containing the same amounts of vitamin-carrying substances as are required for growth.

4. Lactation requirements for vitamin B appear to be 3 to 5 times greater than for growth. The demand for an increased amount of vitamin E is not as clearly defined. There is a progressively increased demand for food during the lactation period.

5. Special attention has been given to the preparation of test animals for the vitamin A studies. Using an irradiated purified vitamin A-free ration, the exhaustion of this vitamin stored in the body has been uniform and rapid. It has been found undesirable to retain such depleted animals for extended periods before therapeutic methods are begun in order to avoid dealing with grossly pathological animals

6. A new method for testing the vitamin A content of foods is described.

7. The effectiveness of the vitamin B-free ration is discussed.

8. Data are presented which indicate the presence of two factors in yeast, instead of the one hitherto recognized vitamin B.

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HUMAN MILK STUDIES.

II. THE QUANTITATIVE ESTIMATION OF VITAMIN A.*

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Although the literature contains references (1-4) to cases of xerophthalmia and other symptoms of vitamin A deficiency in artificially fed infants and in children from 2 to 5 years who have been fed largely upon cereals and skimmed or condensed milks, the authors are not aware of any information concerning the development of these characteristic conditions in babies during breast feeding. Block (3), in discussing war time conditions in Germany, states that, "Xerophthalmia was never observed in children suckled by a mother capable of yielding sufficient milk." In children vitamin A deficiency is not diagnosed as such until advanced keratomalacia and corneal ulcers are apparent. From animal experimentation it is known that corneal ulceration is the outward manifestation of an acute nutritional upset which has been present for some time. All the progressive changes beginning with loss in ability to grow, keratinization of the epithelial tissue, and loss in resistance to disease resulting in generalized infections, are symptoms that can be observed in experimental animals, but are not generally recognized in children as being indicative of vitamin deficiency. Ross (4), in his observation of four cases of xerophthalmia in children, states that, "The late appearance of keratomalacia, which is the only characteristic symptom of this

* Progressive preliminary reports of this investigation were presented to the American Society of Biological Chemists in Cleveland, Ohio, December 29, 1925, and to the American Pediatric Society in Niagara Falls, Canada, May, 1926, see *J. Biol. Chem.*, 1926, lxxvii, 1, *Am. J. Dis. Child*, 1926, xxxiv. 456.

disease, makes it rather difficult to recognize the condition at an early stage." Although the gross pathological symptoms of vitamin A deficiency may not develop, there is no adequate means of determining how many of the symptoms of malnutrition or other disturbances of infancy in either artificially or breast-fed babies are due to a low content of the so called vitamins in the food

From the reports thus far obtained, fresh whole milk either from the cow or human would, therefore, appear to be an adequate source of vitamin A, since babies on either of these nutrients at least have not developed the *advanced* symptoms of xerophthalmia. A given amount of any one vitamin, however, may be sufficient to protect against the macroscopic syndrome of a dietary deficiency, as in the case of corneal ulcers, but may fall short of the amount required to promote optimal nutrition and growth. Milk, no doubt, at times may be deficient in certain food constituents, since by animal experimentation diet has been demonstrated to influence the concentration of vitamins in the milk (5, 6).

Little is known regarding the daily vitamin requirement of man or of his ability to store these factors in his tissues. But in certain clinical cases of xerophthalmia there must have been a very limited storage of vitamin A in the body tissues, because of the very rapid development of xerophthalmia in babies when removed from the breast and given artificial feedings. Schwartz (7) reported the development of xerophthalmia in a baby in 21 days after it had been removed from the breast in its 10th month and placed upon a diet consisting of oatmeal gruel and Mellin's Food in water. Mori (2), in his extensive experience in Japan, found xerophthalmia most frequent in children who were undergoing the transitional period between breast feeding and the mixed dietary. Breast feeding was almost universal for at least 1 year, and the transitional diet consisted practically entirely of cereals and some vegetables, cow's milk and fats being seldom used. These observations would indicate that the diet of the mother during pregnancy and lactation was deficient in vitamin A with a consequent varying amount of this important food principle stored in the tissues of the young. That this is possible has been shown by Kennedy, Palmer, and Schlutz (8), and, indeed, theirs is the only reference to the vitamin A content of

human milk in the literature at our disposal. They found that although 10 cc. of breast milk daily from mothers on an adequate dietary furnished sufficient vitamin A for optimal growth in the rat, a similar quantity of milk from a mother whose diet was distinctly inadequate in both vitamins A and B was wholly insufficient.

From the standpoint of infant feeding a more precise understanding of the quantitative relations of the vitamins eaten and the milk secreted is desirable. An investigation, then, upon the vitamin A content of mixed human milk from women on the average American dietary has been made in order to determine the relative concentration of this vitamin and to establish an index for comparison with data from a subsequent study of the effect of diet upon human milk secretion.

EXPERIMENTAL.

Procedure.

Standardized rats, reared to 21 days of age on the stock diet,¹ have been used as experimental test animals. They were confined to individual metal cages (9), the trays of which were fitted with absorbent paper² held down by flat screens. The basal ration, consisting of 18 per cent casein, 76 per cent dextrin, 4 per cent salt mixture (10), and 2 per cent agar, was endowed with antirachitic properties by a 30 minute exposure to ultra-violet light³ at a distance of 15 inches. The irradiated food and distilled water were fed *ad libitum* while 0.4 gm. of yeast⁴ was given daily to supply the additional factors necessary for growth with the exception of vitamin A. Weekly growth weights and food consump-

¹ The stock diet consisted of two-thirds whole wheat, one-third whole milk powder, and sodium chloride to the amount of 2 per cent of the wheat. The whole milk powder used was Klm, secured from the Merrell-Soule Co., Syracuse, N. Y.

² Absorbent paper obtained from Penobscot Chemical Fibre Co., 211 Congress Street, Boston.

³ Alpine Sun Lamp made by the Hanovia Chemical and Manufacturing Co., Newark, N. J.

⁴ The authors are indebted to Dr. Robert E. Lee of the Fleischmann Yeast Company, 699 Washington Street, New York, and to Dr. M. H. Givens of the Northwestern Yeast Company, 1750 North Ashland Avenue, Chicago, who furnished yeast for these experiments.

tion records were determined. Examination of the vaginal epithelium was made daily for the detection of the persistent cornified cell, one of the earliest symptoms of vitamin A depletion (11).

The growth and physiological reaction of the animals upon the standard vitamin A-free ration has been discussed in detail elsewhere (9). The animals have grown normally for the first 5 weeks at which time the appearance of symptoms of vitamin A exhaustion has been uniform and rapid: the persistence of the characteristic type of cornified epithelial cells in the vaginal tract after 23 (\pm 3) days, the cessation of growth, and the appearance of xerophthalmia as early as 34 (\pm 3) days.

The milk used in the preliminary studies was obtained from the Detroit Wet Nursing Bureau where the milk was expressed under aseptic conditions at least twice daily. The milk was bottled, stoppered tightly, and put immediately on ice. Later, analogous portions were taken from each specimen, pooled, sealed air-tight with paraffin, and delivered fresh daily to the laboratory. The maximum time elapsing between the expression of the milk and the feeding of the rats was 24 hours. No effort was made to control the dietaries of the wet nurses other than the supervision already in vogue at the Wet Nursing Bureau. It was found by a survey that none of the women, ten to sixteen in number, took cod liver oil while supplying milk to this organization. The milk produced by each wet nurse was adequate for normal growth as shown by careful monthly observations of her infant. Milk is not accepted after the 14th month of lactation. It might be of interest in the vitamin A studies to note that the fat content of the pooled milk ranged between 2.7 and 3.5 per cent.

Experimental Data.

In the earlier experiments the prophylactic type of procedure was used. When 2, 5, 10, and 15 cc of human milk daily in conjunction with the standard vitamin A-free ration were fed to 23 day old rats, growth in all cases was normal. A daily amount of 1.5 cc of this substance, however, resulted in normal growth for about 84 days only, with subsequent maintenance of weight (Chart I). This was accompanied by respiratory involvement, as evidenced by continual sneezing, and the presence of hemorrhagic nasal secretions on the tray paper and food cups. There

the vitamin in amounts originally conferred upon it by the maternal parent during gestation and lactation.

Physiological Behavior Other Than Growth.

Following the administration of 2 and 2.5 cc. of human milk to female rats depleted of vitamin A and showing only the characteristic cornified cells in vaginal smears, normal ovulation and the cyclic changes in the vaginal epithelium have been resumed. With these amounts of milk daily the establish-

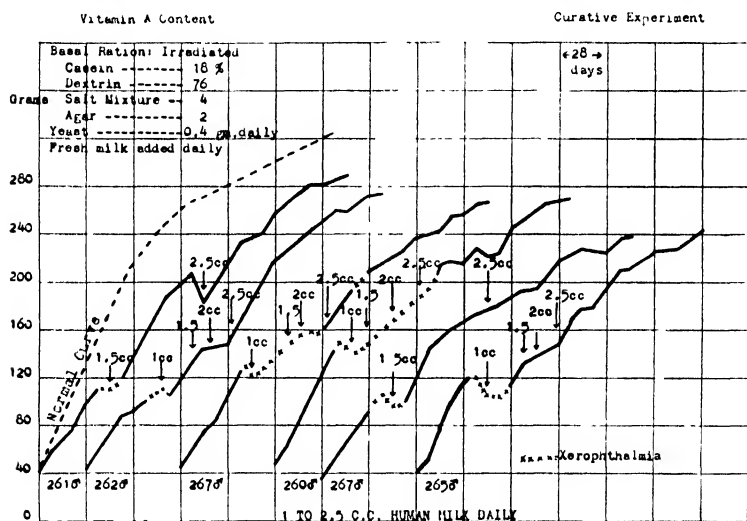


CHART II The ineffectiveness of 1, 1.5, and 2 cc. of human milk in curing xerophthalmia and establishing growth

ment of the normal estrous cycle has taken place in 5 to 7 days. Coincident with this there has been an improvement in appearance and in appetite (Chart IV). Although the renewed growth, physiological behavior, and appearance of the animals have been apparently normal, yet autopsy reports have shown that many of the animals were receiving a minimum amount of vitamin A. In these experiments a very high percentage of the rats at autopsy showed varying respiratory disturbances, such as congested, solid, and anemic areas and nodular growths in the lungs, and a general involvement of the mastoid cells; calculi in the bile ducts were

frequently found in the rats on experiments of longer duration. In spite of the existence of these pathological conditions, the animals appeared to be in excellent nutritional condition with extensive deposits of adipose tissue.

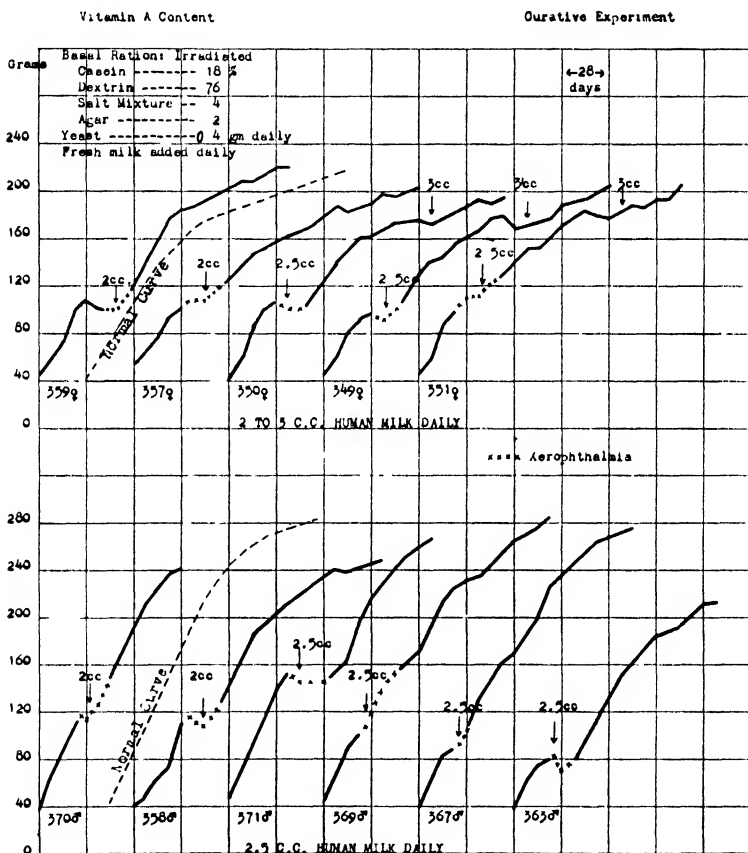


CHART III. Growth curves of rats illustrative of the effect of 2 to 3 cc of human milk in a curative experiment.

Reproduction and Lactation.

In studying the reproductive ability of rats receiving small quantities of human milk daily as the sole source of vitamin A, wheat germ oil, which had been withheld because it deferred the

onset of xerophthalmia for 1 to 3 weeks, was fed in daily amounts of 40 to 50 mg. to supply sufficient vitamin E for normal reproduction. Yeast was given in the same amount as in the growth

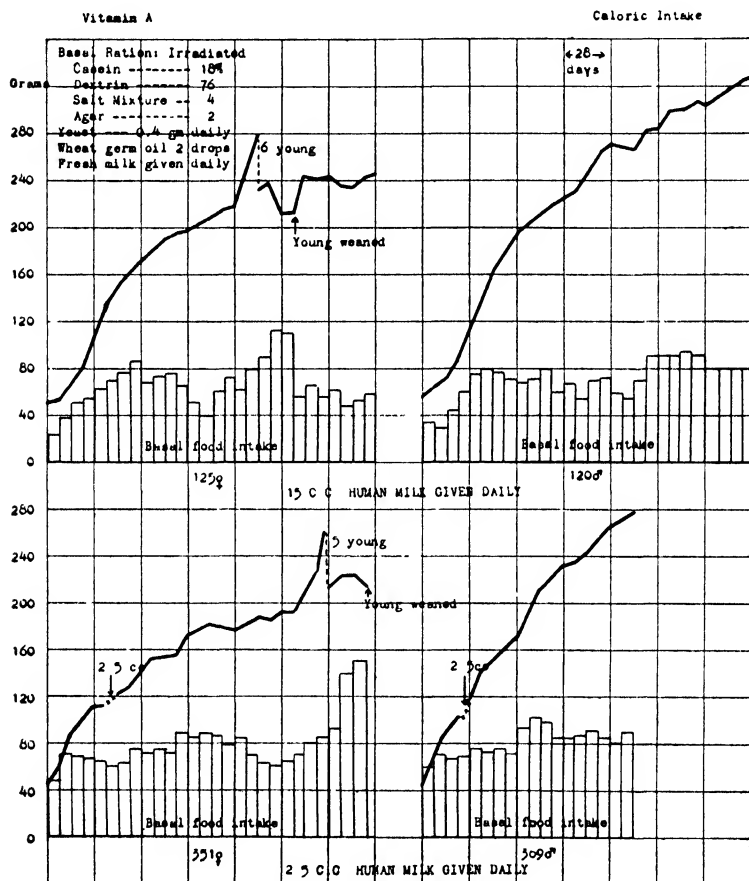


CHART IV Growth of rats and their food intake when 15 cc of human milk serve as the source of vitamin A in a prophylactic experiment and when 2.5 cc. are given in a curative experiment.

studies, *i.e.* 0.4 gm. daily. In the early prophylactic studies the rats receiving 5, 10, and 15 cc. of milk daily from the 23rd day of life reproduced normally. This was likewise true of those re-

TABLE I
Growth of Litters and Food Intake of Rats Receiving Human Milk as a Source of Vitamin A

Rat No	Vitamin additions daily			Young				No found*	Gain or loss during lactation	Mother			Time re-quired for product on of xeroph-thalmin in young on vitamin A-free ration	Remarks
	Human milk	Yeast	Wheat germ oil	Weight			No weaned			Basal food intake				
				7th day	14th day	21st day								
											1st wk	2nd wk		
	cc	drops	drops	gm	gm	gm	gm	gm	gm	gm	gm			
125	15	4 0†	15	11	13	24	34	7	-10	123	135	183	Litter reduced to 7. Pro-phy-lactic experiment.	
124	10	1 5†	12	9	16	28	40	6	+8	99	200	230	"	
129	5	1 5†	10	8	13	27	38	6	-5	174	152	212	"	
216	2	4 0†	15	9	12	24	31	6	+27	101	158	155	"	
264	2	2 0†	10	8	10	18	24	6	+5	67	116	140	Curative experiment	
359	2	2 0†	8	3	12	20	30	3	+15	83	94	99	"	
269	2½	4 0†	10	8	11	24	32	6	+43	93	100	179	"	
351	3	2 0†	8	5	12	23	35	5	-2	126	150	163	"	
353	3	2 0†	8	5	15	26	39	4	-9	98	129	140	"	
350	3	2 0†	8	6	9	19	31	6	-14	67	100	135	"	
349	3	2 0†	10	6	15	25	36	5	-12	66	138	166	"	

* The number of young was reduced to six on the 1st day

† Fleischmann's yeast.

‡ Northwestern yeast

ceiving 2 to 3 cc. from the time xerophthalmia appeared (approximately the 56th day of life) up to and including gestation. The litters have been normal in size, weight, and appearance.

The routine procedure of reducing the number of young in each litter to six, and allowing them to nurse for a period of 21 days only, was followed to provide a standard for comparison with data secured from similar and other types of diets. Since it had been found previously (9) that during lactation rats exhibited a demand for increased amounts of vitamin B and possibly vitamin E, both the yeast and the wheat germ oil were increased in adequate amounts (Table I). In the prophylactic experiments the rats receiving the larger amounts of human milk (5, 10, and 15 cc) were able to nurse and rear their offspring in a normal manner, taking $42 (\pm 7)$ gm., the average weaning weight of young reared on the standard complete ration, as a criterion of normal growth. Rat 216 receiving 2 cc daily produced young somewhat below the average in weight. In the curative type of experiments lactation was less satisfactory, the young from litters of six being definitely subnormal in weight (Table I). The general physical appearance of all the young weaned on these rations, however, was apparently normal. They were active, with sleek and glossy hair. The only evidence of subnormality was their slightly smaller size. From a survey of the food records of the mothers of these young, it is seen that there has been a progressively increased demand for the basal ration, which has been found in a previous study to be concomitant with normal and successful lactation when vitamins were fed in generous amounts (Chart IV).

Perhaps the most interesting data obtained from the entire study on the quantitative estimation of vitamin A in human milk, has been the behavior of these offspring born to the mothers receiving the small amounts of milk daily as the sole source of vitamin A in an otherwise adequate diet. When these *apparently normal* rats, 21 days old, in turn were placed on the standard vitamin A-free ration, their deportment was very striking; instead of the usual average depletion period requiring 34 days, xerophthalmia made its appearance earlier. The young of a rat on 15 cc. of milk daily from the 23rd day throughout life, grew fairly well for 30 days at which time xerophthalmia manifested itself,

while those from the 2 cc. series developed this condition in 21 days. The young of females receiving 2 to 3 cc. of milk therapeutically, however, not only failed to gain normally in weight, but many even ceased to grow after they were weaned. A number died in the 1st week of the experiment without exhibiting gross symptoms of vitamin A deficiency. Most strikingly, the condition of xerophthalmia appeared in the others within 6 to 12 days (Table I). These results are similar to those of Sherman (12), who in studying the relation of the vitamin A content of the diet to the amount of vitamin A stored in the body, has stated that the ability of the mother to lay down this vitamin in the body of the young depends upon her diet up to the maximum absorption ability of the young.

At autopsy these small rats showed advanced stages of keratinization of tissue and consequent abscesses in the upper respiratory and digestive tracts, conditions far more generalized and outstanding than those observed in the standard rats held for 7 to 10 days following the appearance of xerophthalmia in 34 days on the standard ration. Tissues of the tongue, soft palate, and salivary glands contained cavities filled with yellow pus-like material. In every instance there was involvement of at least one mastoid. A very consistent finding at this early stage was the marked atrophic condition of the salivary glands and pancreatic tissue, these glands appearing to be but a clear transparent mass. The small white areas denoting keratinized epithelial tissue of the secreting cells, described by Wolbach and Howe (13), were frequently present. Another striking feature of these rats was the anemic condition of the lungs, liver, and kidneys, denoting a poor utilization of the iron in the basal ration. The advanced degree to which these gross pathological conditions had attained within 2 weeks after removal from the mother's breast to the standard vitamin A-free ration indicates the probability that these changes had begun during lactation, although the rats appeared normal in all respects except size.

This study demonstrates that although 2 to 3 cc. of human milk daily may supply sufficient vitamin A for normal growth and reproductive functions in rats, yet the vitamin A content of the diet is too low for the increased metabolic demands accompanying lactation. This is evidenced by the subnormality, though

slight, in the rats at weaning age, and in the low amount of vitamin A the mother was able to confer to the tissues of her six young, although she appeared to be in good physical condition.

DISCUSSION.

The data on the vitamin A content of breast milk as influencing the economy of nutrition during growth, gestation, and lactation of the rat again emphasize the significance of this food principle particularly during the periods of most rapid growth, *i. e.* fetal, infancy, and childhood. Milk from women on the average American dietary is apparently a relatively rich source of vitamin A, since 2.5 and 3 cc. of the mixed milk from a group of wet nurses in various stages of lactation were sufficient to satisfy the nutritive demands for this factor during growth and reproduction of the rat. Further evidence of the potency of breast milk is illustrated in the children's clinics, where xerophthalmia is rarely, if ever, observed in suckling infants.

Adverse conditions arising during the early periods of life play a profound rôle in the future development of the growing organism, and there is reason to believe that abnormal conditions, such as deficient diet, are even more detrimental when they occur during fetal growth. It is apparent that if there is a deficiency in the food supply of the mother, either the maternal or the fetal tissue, and perhaps both, suffer. Data presented here on a minimum amount of vitamin A for a long period of time illustrate that rats grow at the average rate, females are able to reproduce, and lactation from external appearances of the mother and her young may be quite normal. The crucial point, however, comes with the mother rat's inability to endow her offspring with a normal heritage of vitamin A, as exhibited by the inferior deposition of this substance in their tissues. The studies of Nelson, Lamb, and Heller (14) upon the effect of a diet deficient in vitamin A upon rabbits are significant in this respect. One female rabbit gave birth to five young which she suckled and cared for in an apparently normal manner, but soon after the litter was cast she lost weight and developed xerophthalmia; two young died before their eyes were opened, and two developed xerophthalmia. That these conditions were due to the lack of vitamin A was demonstrated

by adding butter fat to the mother's diet with a consequent alleviation of the condition in both the mother and young.

That a diet low in vitamin A is closely associated with a child's susceptibility to infections (15, 16), certain eye abnormalities (17, 18), and other untoward results is well illustrated in many of the children's clinics (1-4). The faulty diets of the human race are not examples of a single but of multiple dietary deficiencies; a slight insufficiency of any one of these, over a long period of time, may produce effects as serious as conditions resulting from a wholly inadequate diet. The underfeeding of one or all of the vitamins results in serious consequences to the organism, the advanced lesions and symptoms of which are fairly well understood, whereas the full consequences of the border-line conditions are merely conjectures.

The authors take this opportunity of expressing their appreciation of the cooperation of Dr B Raymond Hoobler and of the members of the Detroit Bureau of Wet Nursing of the Woman's Hospital, whose keen interest and enthusiasm have assured the continuous daily supply of fresh human milk. The constructive suggestions offered by Professor E. V McCollum, Research Consultant to the Merrill-Palmer School, have been very helpful

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HUMAN MILK STUDIES.

III. THE QUANTITATIVE ESTIMATION OF VITAMIN B.*

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In previous investigations it has been found that the vitamin concentration of milk is dependent upon the vitamins ingested in the food of the mother, the maternal organism being unable to synthesize these factors (1). This fact has been confirmed by a number of observers upon several species of animals and there remains little doubt that this phenomenon holds true for all alike, although there are few quantitative confirmatory results for man. Exceedingly little data are available on the vitamin content of human milk using the newer analytical testing technique, with a constant supply of milk collected and fed under carefully controlled conditions in accordance with our recent knowledge of experimental feeding methods.

The vitamin B content of human milk has been studied to a limited extent only as revealed by a study of the literature. Andrews (2), a pioneer in the field, had sixteen puppies nursed by mothers whose babies had died of beriberi. Within 3 or 4 weeks all the puppies exhibited incoordination and weakness of the extremities and finally died. This work was of value primarily from a qualitative standpoint. Andrews concluded from his observations that, "In the Philippines the mortality is greatest among breast-fed children, possibly because of the poor quality of mothers' milk. The latter is probably deleterious, by reason of what it lacks rather than because of any harmful constituent. . . . It seems probable that there is an intimate

* Progressive preliminary reports of this investigation were presented to the American Society of Biological Chemists in Cleveland, Ohio, December 29, 1925, and to the American Pediatric Society in Niagara Falls, Canada, May, 1926; see *J. Biol. Chem.*, 1926, lxxvii, p li; *Am. J. Dis. Child.*, 1926, xxxiv, 456.

relation between beriberi of infants and a mother's milk poor in quality and lacking certain necessary elements which are not included in the mother's dietary." Quantitatively, Gibson and Concepción (3) found 20 cc. of human milk insufficient to prevent birds on a polished rice diet from developing polyneuritis. Gibson (4), in discussing Vedder and Clark's work (5), stated that "It would seem if Vedder's statement that fowls kept on 5 cc. fresh cow's milk with milled rice are partially protected be accepted, that normal human milk must contain not more than one-fourth of the amount of the vitamin of the former." When fed as the only source of vitamin B in a ration otherwise adequate, Osborne and Mendel (6) obtained sub-normal growth in rats receiving 5 cc. of human milk daily, and only fairly good growth when the desiccated milk was fed in an amount equivalent to 10 cc of whole milk daily. In summarizing their observations they (7) wrote "Incidentally an opportunity has been afforded during the past year to make a few feeding experiments with human milk with a view to testing its potency as a source of water-soluble vitamin. From the few limited data thus far secured there is no evidence of a greater content of this food factor in this important nutrient than in cow's milk." More recently Kennedy, Palmer, and Schlutz (8) have demonstrated that 10 cc of breast milk daily as the sole source of vitamin B were inadequate for the production of normal growth of the rat.

Since all new born mammals are nourished with milk, and since the human infant is dependent upon and sustained through the 1st year of life on a diet consisting in a great part of milk, it is essential for optimal growth of the child to have a liberal supply of milk of adequate quality. To supplement the knowledge gained from experiments on animals in which diet has been demonstrated to influence the quality of milk produced, a series of studies upon the factors influencing human milk production have been begun. This paper deals only with one limited phase of the problem; namely, the quantitative estimation of the vitamin B content of composite samples of fresh, certified milk as produced by a group of ten to sixteen healthy women in various stages of lactation, on the average American dietary. These data are preliminary to a subsequent investigation concerning the effect of diet upon human milk production.

EXPERIMENTAL.

Procedure.

Emphasis has been placed upon the importance of using animals of known heredity and reared on a fixed dietary in all quanti-

tative vitamin studies. Rats, therefore, from a carefully selected colony which has been kept under standardized conditions have been used as experimental subjects to which the breast milk has been fed. Details of the methods used in the standardization of the animal colony and the technique employed in the quantitative vitamin studies in this laboratory have been discussed elsewhere (9). In the vitamin B studies, both curative and protective experiments were conducted; but, since rats do not eat human milk with avidity, more satisfactory consumption of the larger quantities was obtained when the animals were deprived of vitamin B for a short period of time before administering the milk. Young rats, therefore, 21 to 26 days old, were retained on a vitamin B-free ration for 2 weeks, at the end of which time most of the animals had stopped growing and all were eager to consume the daily allotment of milk, even in quantities as great as 30 cc.

In the vitamin B experiments, the purified basal ration (9) consisted of 18 per cent casein, 76 per cent dextrin, 4 per cent salt mixture,¹ and 2 per cent agar-agar, together with 5 drops of cod liver oil² (100 to 120 mg) and 2 drops of wheat germ oil (40 to 50 mg) to supply the vitamins A, D, and E. All animals were kept in individual cages on raised screens; the basal ration and fresh clean water were available at all times. The vitamins and milk were fed in accurately measured quantities fresh daily in separate dishes apart from other food material, thus insuring a quantitative determination of each food ingested by the individual animals without the possibility of the vitamins or the milk influencing the flavor of the basal ration. Weekly growth increments were ascertained and wherever it seemed necessary more frequent observations were made. Daily routine vaginal smear examinations were made and when possible, the ability of the female to bear and rear young was tested.

Experimental Data.

In the estimation of the vitamin B content of breast milk, as well as in other vitamin studies (10), the optimal degree of excellence in growth and physiological well being of the rat at

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 557.

² Cod liver oil No. 1610, Mead Johnson Company, Evansville, Ind.

various stages of its existence has been taken as the standard for judging the adequacy of any given quantity of milk when fed in conjunction with a diet complete in respect to all known dietary

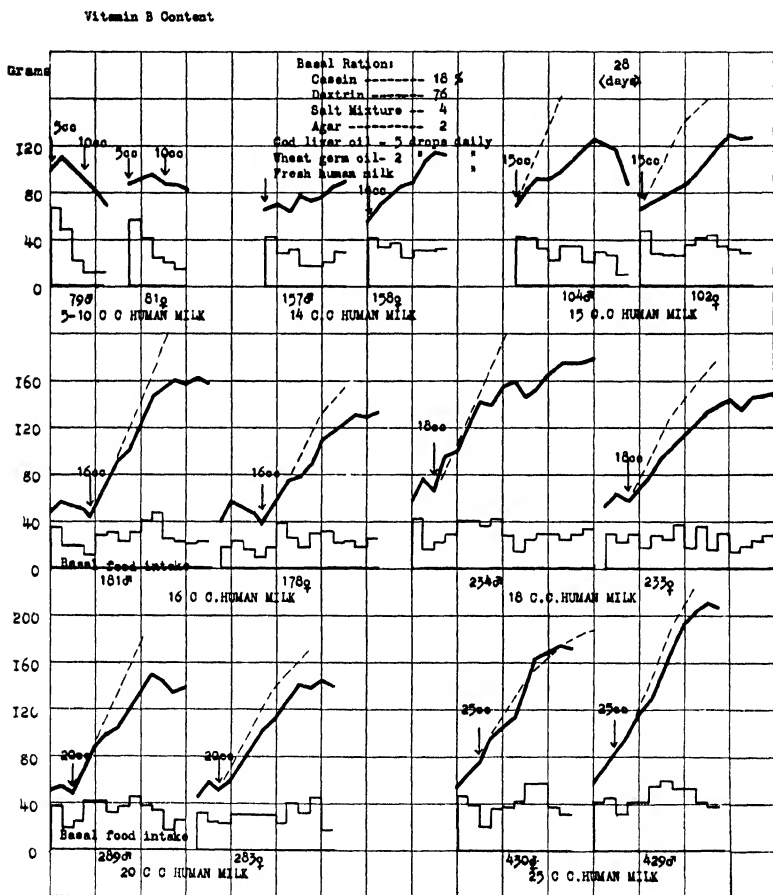


CHART I. Growth of rats on different amounts of human milk as the only source of vitamin B The basal food records are also recorded

essentials with the exception of the one vitamin under investigation.

In no instance, under conditions prevailing in this laboratory, has it been possible to secure average growth and normal physio-

logical behavior when 5, 8, 10, and 12 cc. of fresh mixed human milk served as the only daily source of vitamin B in a diet otherwise complete. Whereas on quantities of 14 and 15 cc. subnor-

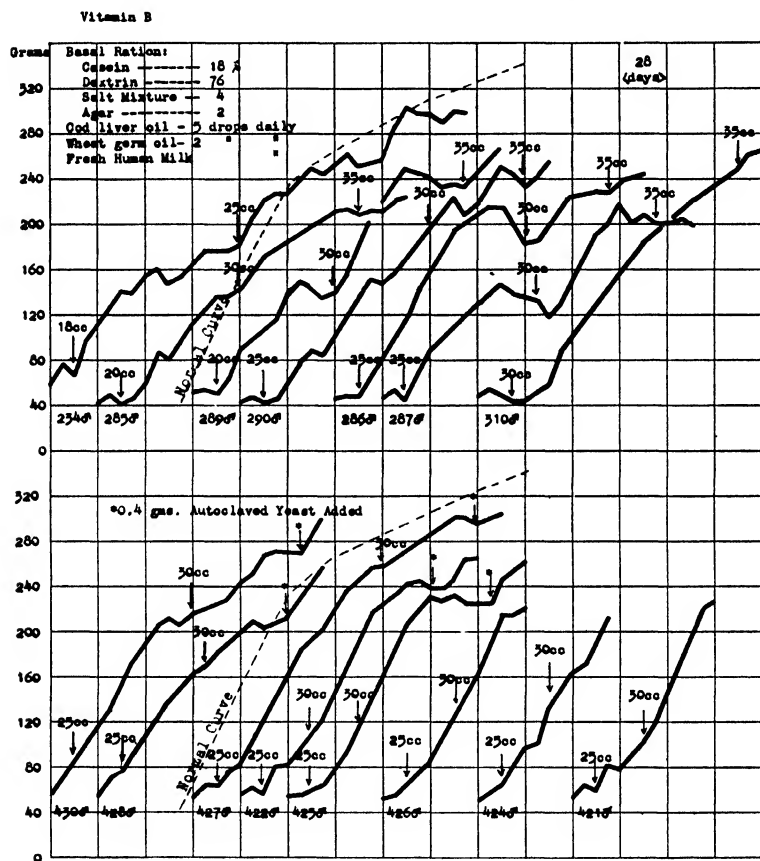


CHART II A group of representative growth curves of rats illustrating the behavior of rats upon different quantities of human milk as the only source of vitamin B in a diet otherwise complete

mal gains were made, it was not until 16, 18, and 20 cc.-of milk were given daily that continuous growth, although below the average, was secured (Chart I). But even with such large quanti-

ties of milk there was a tendency to delayed sexual maturity in the females receiving levels below 18 cc. daily; normal ovulation has taken place only when a minimum of 20 cc. served as the source of vitamin B. Higher levels of 25 and 30 cc. of human

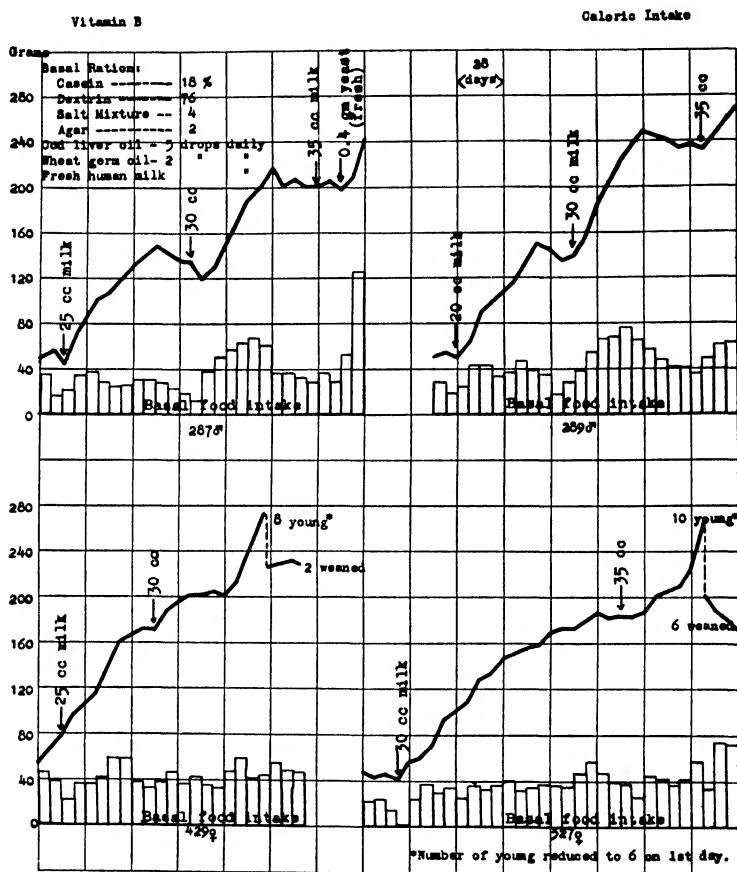


CHART III. Growth and food intake of rats on a diet in which human milk furnishes vitamin B

milk were sufficient to furnish enough of this important dietary component for normal growth and sexual activity in the young animal, while 35 cc. were necessary to produce continuous growth in larger rats weighing 200 to 240 gm. (Chart II).

The rats, though litter mates, have not grown as uniformly on the low amounts of human milk as they have on other vitamin B-supplying substances such as yeast (11) or cow's milk, and it is consequently impossible to correlate the maximum weight attained and the amount of milk consumed. These results cannot be explained on the basis of a seasonal variation in the vitamin content of the milk. The causal factor may be a possible variability in the vitamin B content of the milk from day to day, a possible incomplete assimilation of this vitamin due to the diarrheal condition on levels above 14 cc., or an actual variation in the amount of basal food eaten (12). The 1:2.5 ratio between the amount of basal food and the milk, which Johnson (13) found necessary to normal growth when feeding cow's milk, is not as definite in the human milk studies. More human milk than cow's milk is required per unit of basal food for normal growth. Consistently less basal food has been eaten than when the same level of cow's milk has been fed, again indicating a paucity of vitamin B in human milk. On a given amount of milk the rats have grown for a short period after which the growth curve has flattened and ultimately reached a plateau. When the quantity of milk has been increased, growth has been resumed. Coincident with this there has been a greater consumption of calories in the form of the purified basal ration (Chart III). It is difficult to conceive that the increase of 5 cc. of milk alone in so far as food nutrients other than the accessory substances classified as vitamin B are concerned is sufficient to stimulate so marked a response in growth and appetite. The only growth curves consistently uniform and normal, and within the limits of variability of animals on the standard complete ration (9) were those exhibited by rats receiving an initial large amount of milk daily (25 cc.) with the quantity increased as growth proceeded (Chart II).

Cowgill, Smith, and Beard (14) have suggested that vitamin B is closely related to metabolism in the adult mouse, rat, and dog, and have correlated the body weight and caloric intake per day with vitamin B requirement. They also found that during growth some other factor, as yet undefined, appears to affect the vitamin requirement, since during the rapidly growing period the vitamin requirement is much higher. Karr (15) has concluded

from a series of experiments that "some relation exists in the dog between the desire to partake of food and the amount of the so called water-soluble B vitamin ingested." Kennedy and Dutcher (16) believe that "the effect of vitamins is not necessarily an excitant to appetite, but rather a stimulation to metabolic processes which promote growth and normal functioning." Gross (17) has found that in vitamin B deficiency there is a lowered metabolism with an accompanying low body temperature. The probable *modus operandi* is that vitamin B stimulates the metabolic processes and in turn the appetite is increased due to a greater demand for food to satisfy the increased metabolic activity of the protoplasm.

The studies of Goldberger and Lillie (18) and Smith and Hendrick (19) would indicate that there are two factors involved in vitamin B studies. It has been shown that yeast heated at 15 pounds pressure for 3 hours neither produces growth nor prevents polyneuritis, but when fed in conjunction with the antipolyneuritic factor it does stimulate growth (9). Autoclaved yeast in 0.4 gm. quantity daily has, therefore, been fed to a few rats that had ceased to grow on a large quantity of milk to determine whether an increment in weight could be secured. In the majority of these cases there was a definite response manifesting itself in increased growth as shown in Chart II. From these observations it would appear that growth on large amounts of human milk daily is not optimal in the absence of some substance in yeast which is definitely not concerned with the antipolyneuritic vitamin. If this substance in autoclaved yeast is identical with the pellagra-preventive factor of Goldberger and associates, then *human milk* must be a relatively poor source of this vitamin.

Physiological Behavior of Rats on Large Quantities of Breast Milk.

It has previously been stated that although young rats on 16, 18, and 20 cc. of breast milk as the only source of vitamin B in an otherwise complete diet made gains in weight which almost approximate the average growth curve, there was a tendency to delayed sexual maturity in the females on levels below 18 cc. daily. Normal ovulation took place only when a minimum of 20 cc. of breast milk furnished the vitamin B. With higher levels of milk, *i.e.* 25, 30, and 35 cc., normal physiological function was

attained. That this was possibly due to an increase in vitamin B has been shown by Evans and Bishop (20) who demonstrated that the diet must contain a sufficient amount of vitamin B before there can be normal physiological function of the sexual organs. A deficiency is evidenced in delayed sexual maturity of the females and a definite disturbance in the estrous cycle.

When a sufficient amount of human milk was given to satisfy the vitamin B requirement, the rats grew and attained average weight, although at the same time they exhibited a somewhat unkempt appearance, the hair appeared coarse, and the body was less firm than the controls of the same age on the experimental diet with yeast as the sole source of vitamin B. Liquid or soft and unformed feces were excreted on levels of milk above 14 cc daily throughout the period of milk ingestion. Lactose has been assigned as the causal factor by Hamilton and Card (21), who found pure lactose an irritant to the gastrointestinal mucosa of the chicken, and by Sure (22), who observed a similar transitory condition in rats fed on a diet containing large quantities of dried cow's milk. Human milk carries a high percentage of lactose, appreciably higher than cow's milk, but it is very difficult to determine how many of the symptoms are due to the digestive products of the sugar and how many are due to the change in bacterial flora of the intestine to the fermentative type.

Reproduction and Lactation.

Observations concerning the effect of human milk as a source of vitamin B upon reproduction are of interest. Sexual maturity, though late, has been attained and normal ovulation has taken place when a minimum of 20 cc. of breast milk was given daily in a diet composed of all factors essential for growth, reproduction, and lactation other than vitamin B. Reproduction has taken place on a minimum of 25 cc. Litters born to mothers receiving 25 cc. and even 30 and 35 cc. of milk have been normal in number and appearance, but lactation has been uniformly unsuccessful. The number of young per litter has been reduced to six on the 1st day after birth. Lactation was established as determined by the appearance of milk in the stomach of the young, but as early as the 7th day the young were subnormal in weight, appearance, and

TABLE I.
Growth of Litters and Food Intake of Rats Receiving Human Milk as a Source of Vitamin B.

Rat No	Vitamin additions daily			Young				Mother				Remarks.	
	Human milk	Wheat germ oil	Cod liver oil	No found *	Weight			Gain or loss during lactation	Basal food intake				
					7th day	14th day	21st day		No weaned	1st wk	2nd wk		3rd wk
	cc	drops	drops		gm	gm	gm	gm	gm	gm	gm		
232	25	15	5	4	14	20	26	-3	120	129	150	Shavings used for bedding	
233	25	15	5	9	12	21	27	-19	66	137	130	" " " 1 young eaten on 2nd day	
249	30	25	5	7	12	27	38	+6	96	81	70	Shavings used for bedding. 4 young eaten on 2nd day	
247	30	25	5	9	7	9	12	+9	49	53	82	Shavings used for bedding. 1 young dead on 14th day, 2 young dead on 19th day.	
284	30	25	5	8	11	22	29	-7	90	114	128	Shavings used for bedding. 1 young dead on 12th day, 1 dead on 20th day.	
292	30	15	5	9	12	18	23	-17	55	90	75	Shavings used for bedding 1 young dead on 3rd day.	
312	30	10	5	11	13	21	26	-39	45	70	80	Raised screens	
429	30	10	5	8	8	14	20	-30	52	53	41	" " 2 young eaten on 4th day, 1 killed on 6th day; 1 dead on 8th day	
431	30	10	5	8	11	15	20	+34	55	46	63	Given only 3 young, 1 eaten on 2nd day, 1 eaten on 6th day. Raised screens.	
327	35	10	5	10	12	19	22	-24	41	81	72	Raised screens.	
288	35	15	5	7	9	18	22	-37	72	69	66	" " 1 young dead on 12th day, 1 killed Very emaciated.	

* All litters reduced to six on 1st day.

behavior. The inability of the mother to care for her offspring was demonstrated by the fact that the young were undersize, their hair developed slowly, their skins were wrinkled, and their appearance was unkempt. A marked characteristic was the unusual nervousness and unrest that existed among the individuals in the litter, a condition which was alleviated by the addition of yeast to the diet of the mother. The mortality of the young was very high since many died and others were eaten by the mothers. In only one instance was a mother able to nurse six young for 21 days (Table I). The young that survived the 21 day nursing period were few in number and were subnormal. It is possible that there was a lowered milk output by the mother, as well as a deficiency in vitamin B. Whether the findings of Andrews (2) that women with beriberi were able to produce considerable quantity of milk, but of inferior quality, holds true for these rats is questionable, since as yet there are no adequate methods for determining the amount of milk secreted by rats.

It has been shown in previous work (9) that the vitamin B requirement of the lactating rat is 3 to 5 times its growth needs and that there is a progressively increasing demand for the basal ration. Table I shows the extremely low, and in some cases, the decreasing amount of food eaten during lactation when 25, 30, and 35 cc. of human milk supply vitamin B. This fact, as well as the inability of the rats successfully to wean their young on large quantities of human milk, may be taken as further evidence of the low vitamin B content of this nutrient. It can further be concluded that the mother's ability then, to supply to her young, whether unborn or born, this important food constituent depends upon the vitamin B ingested in the mother's food. The data obtained from this study of rats during lactation are, therefore, in accord with those of McCollum and his associates (23, 24) who found that vitamin B passes into the mother's milk only when it is present in the diet of the mother.

DISCUSSION.

From the data presented in this paper, it is concluded that the antineuritic potency of mixed human milk, at least from a group of women receiving the average American dietary, is very slight. They demonstrate that the rat is neither able to store an appreci-

able amount of vitamin B, nor to synthesize it in the tissues. The large quantities (25, 30, and 35 cc. daily) of breast milk sufficient for growth of the rat have been found insufficient to satisfy all the demands of the mother and her young for vitamin B during the active metabolic period of lactation and rapid growth period of her young.

In view of the facts presented here, it is possible that many mothers do not supply enough of vitamin B to their babies. At the best, the average healthy mother is producing a milk that is exceedingly low in vitamin B, and for the economy of the mother's nutrition and to safeguard her offspring, food materials rich in this important food component should form a prominent part in the diet of pregnant and lactating women. Food substances rich in vitamins, furthermore, should be introduced early in the nursing period of the infant, whether it be breast- or artificially fed.

The authors take this opportunity of expressing their appreciation of the cooperation of Dr. B. Raymond Hoobler and of the members of the Detroit Bureau of Wet Nursing of the Woman's Hospital whose keen interest and enthusiasm have assured the continuous daily supply of fresh human milk. The constructive suggestions offered by Professor E. V. McCollum, Research Consultant to the Merrill-Palmer School, have been very helpful.

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HUMAN MILK STUDIES.

IV. A NOTE ON THE VITAMIN A AND B CONTENT OF COW'S MILK.

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Beginning with the report of Hopkins in 1912 (1) on the growth-promoting value of small quantities of cow's milk in synthetic diets, the literature contains many papers on the importance of this nutrient as a source of certain factors required for growth and maintenance. Yet the data from different laboratories are difficult of comparison, because of the variation in experimental technique and growth standards and because of the rapidly changing status of our knowledge of vitamins. For instance, Osborne and Mendel (2) and Johnson (3) have demonstrated that at least 16 cc. of cow's milk were required to supply adequate vitamin B to the growing rat, *i.e.* 8 times the amount reported by Hopkins (1); Dutcher and associates (4) likewise, have found that 2 cc. of milk fed as the source of vitamin A with an irradiated ration was as satisfactory for growth as 10 cc. in the absence of the antirachitic factor (5). Since it was of particular interest and importance to determine the comparative vitamin values of milks used in infant feeding, and since the standards in this series of investigations vary somewhat from those used in other laboratories, a study of the vitamin A and B content of cow's milk has been made under the identical experimental conditions maintained in the human milk studies (6).

Raw certified cow's milk,¹ comparable to the human milk pre-

¹ The milk was purchased from the Walker-Gordon Laboratories of the Detroit Creamery Company and received fresh daily in tightly stoppered bottles packed in ice. We are indebted to this company for information regarding the care and feeding of the dairy herd.

vously studied, was selected for this investigation. It was produced under the best of hygienic and nutritive conditions and had the additional value of being the kind recommended in artificial infant feeding. This milk represented a composite from a herd of Holstein cows, 450 in number, fed a monotonous unchanging standard dairy ration consisting of ensilage and alfalfa hay together with a concentrate containing 600 pounds of corn gluten feed, 400 of bran, 400 of hominy, 300 of ground oats, 200 of steamed bone meal, and 20 of salt. A possible seasonal variation in the vitamin A and B content of the milk due to a change in the food of the cows was, therefore, eliminated.

Vitamin A in Cow's Milk.

In this study the standard test rats, 21 to 26 days old, were first depleted of their store of vitamin A by feeding them an irradiated vitamin A-free ration.² At the first appearance of xerophthalmia and failure to grow, certified cow's milk was fed separately in amounts of 1.5, 2, 2.5, and 3 cc daily. On all levels of milk growth was resumed, but the most satisfactory results were obtained when the rats received 3 cc. daily (Chart I). At the end of the experimental period all rats, with the exception of the two showing persistent xerophthalmia, appeared to be in good physical condition, but upon detailed examination at autopsy either single or double mastoid involvement was found in 77 per cent of the cases. The results indicate that, although 3 cc. of fresh raw cow's milk daily may contain adequate vitamin A to produce satisfactory growth in the rat, this small amount does not always protect against secondary pathological conditions.

Vitamin B in Cow's Milk.

The vitamin B content of cow's milk was determined by standardized methods (6) in which rats, 21 to 26 days of age, were held on raised screens and fed the standard vitamin B-free ration²

² In these experiments the basal ration, fed *ad libitum*, consisted of 18 per cent casein, 76 per cent dextrin, 4 per cent salt mixture (Osborne and Mendel), and 2 per cent agar. In the vitamin A studies this ration was exposed to ultra-violet light for 30 minutes at a distance of 15 inches. A daily amount of 0.4 gm. of yeast was fed separately. In the vitamin B studies 5 drops of cod liver oil and 2 drops of wheat germ oil were fed fresh daily apart from the basal ration.

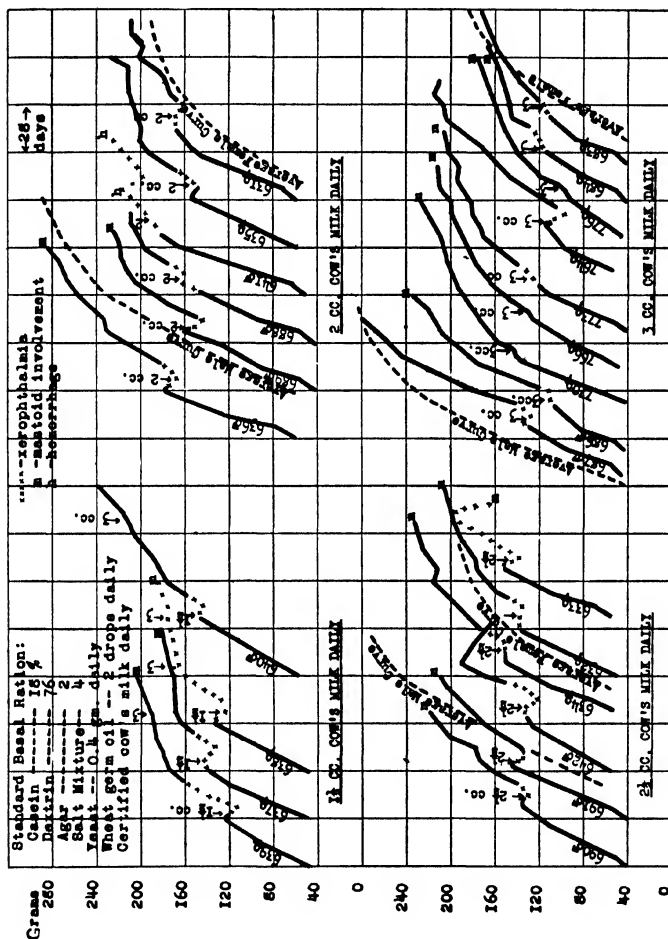


CHART I. The deportment of rats in a curative type of experiment when 1 5, 2.0, 2.5, and 3 0 cc. of cow's milk daily served as the only source of vitamin A in an otherwise adequate ration. Although growth was satisfactory when the higher levels of milk were fed, nasal hemorrhages and pus in the mastoids were encountered in many cases.

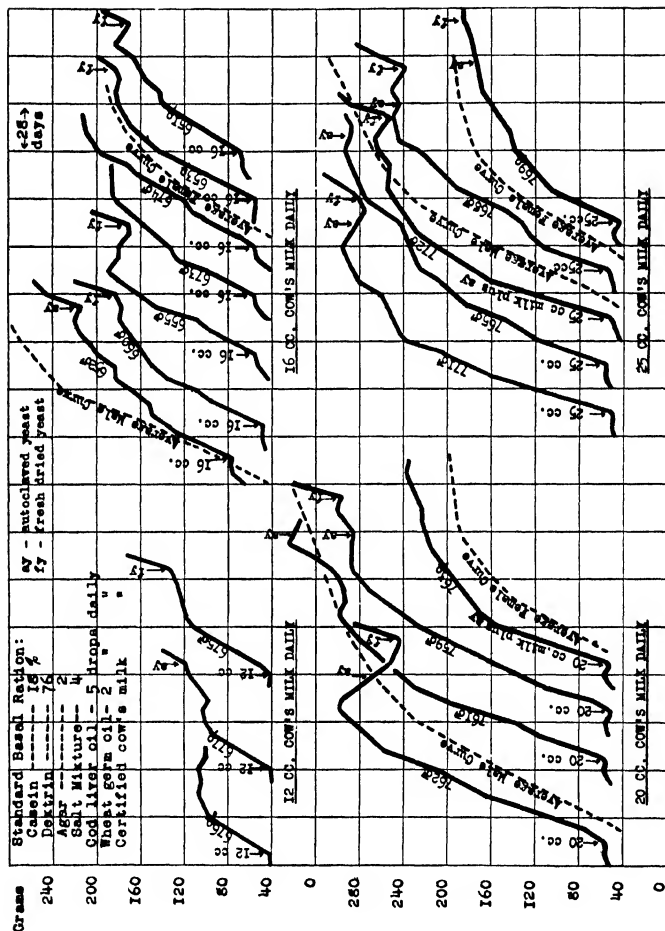


CHART II The growth of rats on a diet in which 12, 16, 20, and 25 cc. of cow's milk daily furnished vitamin B. After the cessation of growth on the higher levels of milk the addition of fresh dried yeast, in contrast to autoclaved yeast, produced a marked increment in body weight.

until growth ceased. Upon the addition of 12 cc. of certified cow's milk daily, the increment in weight was normal for 4 weeks only (Chart II), whereas 16 cc. produced satisfactory growth for a period of 8 weeks. A daily quantity of either 20 or 25 cc., however, allowed for excellent growth, average or above, for about 12 weeks, at which time growth was interrupted. The daily feeding of 0.4 gm. of autoclaved yeast produced no change in the deportment of the latter group; in contrast, the same quantity of fresh dried yeast brought about an immediate response in growth. This

TABLE I

Basal Food Intake of Rats Receiving Cow's Milk as Source of Vitamin B.

16 cc daily			20 cc daily.			25 cc daily		
Rat No.	Average weekly food intake	Ratio food milk	Rat No	Average weekly food intake	Ratio food milk	Rat No	Average weekly food intake	Ratio food milk
	gm			gm			gm.	
653 ♀	43 2	1:2 5	763 ♀	48 8	1:3 1	769 ♀	38.4	1:4 5
651 ♀	35 0	1.3 2						
674 ♂	41 8	1:2 6	761 ♂	63 0	1:2 2	771 ♂	69 7	1:2 5
673 ♂	36 0	1.3 1	762 ♂	59 0	1 2 3	768 ♂	49 5	1:3 5
655 ♂	41 0	1:2 7	759 ♂	55.5	1:2 5	772 ♂	61 6	1:2 8
660 ♂	36 4	1.3 0				765 ♂	62.0	1:2 8
652 ♂	45 2	1:2 2						
Average for ♂ .	40 1	1:2 7	♂	59 1	1:2 3	♂	61 7	1:2 8

would indicate that, of the two factors associated with the growth-promoting properties of vitamin B, the thermostabile fraction is the limiting factor which prevents rats from attaining the average adult weight.

The weekly basal food intake of these rats has been averaged for the period beginning with the addition of milk and ending at the cessation of growth, and is recorded in Table I. From a survey of this table it is noted that the rats receiving 25 cc. of milk daily ate, as an average, approximately the same amount of basal food as was consumed by those given but 20 cc. The data obtained from feeding these amounts of milk to rats as a source of vitamin

B show that the ingestion of large quantities of milk causes no diminution in the amount of basal food eaten.

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THE INFLUENCE OF IRRADIATION UPON OXIDATION PRODUCTS OF CHOLESTEROL.

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It has been shown simultaneously by Steenbock, Black, Nelson, Nelson, and Hoppert (1) and by Hess and Weinstock (2) that cholesterol, in itself inactive, becomes a potent antirachitic agent after irradiation by ultra-violet light. Since this announcement (1925) many investigations have been carried on to determine what the nature of the change is which occurs in cholesterol when it is irradiated. Steenbock and his coworkers (1) considered it very improbable that the potency was resident in any contaminating substance, because highly purified cholesterol—purified by recrystallization from eight to ten times from alcohol and then as the benzoate and acetate from which it was recovered after saponification—could be activated by irradiation. Hess, Weinstock, and Sherman (3) have also shown that it is cholesterol and not an impurity which is affected and that both the anhydrous and hydrated forms of cholesterol and likewise its acetate can be activated. This is evidence that the double bond in the molecule is a prerequisite for activation. Dihydrocholesterol, which lacks the unsaturated carbon linkage does not acquire antirachitic properties when it is irradiated (4). While our research was in progress Rosenheim and Webster (5) stated that both the secondary alcohol group and the unsaturated carbon linkage of the sterol molecule must be intact for the development of antirachitic activity through the influence of ultra-violet light.

It seemed probable that the ultra-violet light might cause a reaction to take place at the double bond, possibly an oxidation. α - and β -cholesteryl oxides, α -cholestantriol, and hydroxycholesterol, simple oxidation products of cholesterol, were therefore prepared in a pure state.

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α-Cholesteryl oxide was prepared from cholesterol (Hibbert and Burt (6) modification of the Westphalen method (7)).

β-Cholesteryl oxide was prepared from cholesterol (Westphalen (7)).

α-Cholestantriol was prepared from cholesteryl acetate (Pickard and Yates (8)).

Hydroxycholesterol was prepared from cholesterol (Lifschütz (9)).

These compounds, irradiated and non-irradiated, have been fed to rats to test their potency. The monobenzoate of cholestandiol was previously made in this laboratory and fed to rats on a rachitic diet before purification (10). As no decidedly positive test was obtained, it was not considered important to spend further time on its preparation. The above products were obtained through the use of mild oxidizing agents, benzoyl hydroperoxide, benzoyl peroxide, and hydrogen peroxide. If irradiation does cause an oxidation of cholesterol, one would not expect a very deep seated change. Therefore, the milder the oxidizing agent, the simpler the resulting product and the more likely that such a compound could be obtained by the irradiation of cholesterol with ultra-violet light.

α- and *β*-cholesteryl oxides are produced simultaneously by oxidation with benzoyl hydroperoxide. They are probably stereoisomers, differing from cholesterol by the addition of 1 oxygen atom, the carbon to oxygen ring replacing the double bond. *α*-Cholestantriol, the product of the oxidation of cholesterol by means of concentrated hydrogen peroxide, perhydrol, is related to *α*-cholesteryl oxide in that it results from the opening of the carbon to oxygen ring by hydrolysis. It differs from dihydrocholesterol in that the 2 hydrogen atoms added to the double bond are replaced by hydroxyl groups. When oxidation with benzoyl peroxide is carried out in glacial acetic acid solution, a different product results, hydroxycholesterol, an isomer of *α*- and *β*-cholesteryl oxides, but quite different in properties, since it gives the reactions of an unsaturated dihydroxyl compound.

EXPERIMENTAL.

Rats weighing 30 to 50 gm. were put on the Steenbock and Nelson rachitogenic diet No. 2966 when they were 28 days old.

	per cent
Yellow corn	71
Wheat gluten	20
Gelatin	5
Calcium carbonate	3
Sodium chloride	1

They received this diet for 24 to 28 days and then in addition the substance to be tested for 10 days. The food intake of the rats was recorded and the animals weighed every 3rd day. All rats that lost weight during the test period or ate less than 3 gm. of food per day were discarded.

The compound to be tested was not incorporated in the diet, but was administered daily to each rat in a solution of linseed oil by means of a pipette. One group of rats received the compound as prepared, another received some of the material irradiated. A quartz mercury vapor lamp (Burdick 55 to 75 volt, 8 amperes) at a distance of 2 feet was used. The oxidation compounds were spread on the surfaces of crystallizing dishes in thin films and the process was carried out in air for 30 minutes.

The Shipley line test, roentgenograms, and blood serum phosphorus (inorganic) determinations (11) were used to determine whether any healing of rickets had occurred. Table I gives a summary of the rat protocols.

DISCUSSION.

α - and β -cholesteryl oxides, hydroxycholesterol, and α -cholestantriol when fed to rats in doses of 3 to 5 mg. per day did not heal rickets. These same oxidation products could not be activated by irradiation. This result is of interest as it definitely eliminates certain oxidation products of cholesterol as antirachitic factors. It is also of interest to note that only slight changes have been made in the original cholesterol molecule but the power of activation by light has been destroyed. Further evidence is also furnished of the importance of the double bond in activation. The oxidation compounds studied in this research are representative of the very simple types of oxidation and none of them are potent antirachitic agents. It seems in the light of Rösenheim and Webster's (12) recent work that it is not cholesterol but a substance which they call provitamin which is changed by irradiation.

TABLE I.

No. of rats	Ration.	Test compound	Dose	Rickets		Blood serum phosphorus, inorganic
				Röntgeno-grams	Lane test	
			mg			mg per cent
3	2966			+	+	1.4
5	2966 + 2 per cent cod liver oil			-	-	7.6
3	2966	Cod liver oil 2 per cent		-	-	5.1
4	2966	Linseed oil 0.1 cc daily		+	+	2.0
6	2966	α -Cholesteryl oxide.	3	+	+	3.3
6	2966	" "	5	+	+	3.2
7	2966	" " (irradiated dry).	3	+	+	3.5
6	2966	" "	5	+	+	2.6
6	2966	β -Cholesteryl oxide.	3	+	+	2.9
4	2966	" "	5	+	+	2.0
3	2966	" " (irradiated dry)	3	+	+	2.4
5	2966	" "	5	+	+	2.5
6	2966	Hydroxycholesterol.	3	+	+	2.3
5	2966	" "	5	+	+	2.6
2	2966	" " (irradiated dry)	3	+	+	
4	2966	α -Cholestantriol	3	+	+	3.2
3	2966	" "	5	+	+	1.5
7	2966	" " (irradiated dry)	3	+	+	3.0

tion. This also supports the earlier conclusion by Schlutz and Morse (13) that an impurity in cholesterol is the potent factor.

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SOLUBILITIES OF THE ANTISCORBUTIC FACTOR PRESENT IN LEMON JUICE.*

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The experimental details summarized in this paper were carried out a number of years ago as a preliminary investigation to what was hoped to have been an intensive study on the composition of the antiscorbutic vitamin. The work was never completed because of the separation of the authors by transfer to other stations

Lemon juice was used as a source of the vitamin in all our experiments. Young guinea pigs weighing from 200 to 300 gm. were used for testing the efficiency of the extracts. Their previous diet included sufficient fresh green vegetables so that there was no danger of incipient scurvy. Their scorbutic diet consisted of an abundance of timothy or clover hay, whole oats, rolled oats, canned evaporated milk, and water.

The lemon juice was partially neutralized by the addition of 50 gm. of calcium carbonate per liter of juice and, without filtration, evaporated under an electric fan in a chamber through which dry air was circulated. The crumbly residue was extracted with absolute alcohol, using 500 cc of alcohol for each liter of original juice. After evaporation of the alcohol the residue was taken up in distilled water. Such extract was always found to be curative.

In place of absolute ethyl alcohol the following solvents were

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substituted with results as indicated. Solvents in each case were carefully purified and made anhydrous before use:

Acetone.	..	Extract	Residue.
Ethyl acetate		Inactive.	Active.
“ “ 75 per cent	}	“	“
“ alcohol 25 “ “		Active.	
Amyl alcohol	.	Inactive.	
Benzyl alcohol.		Active	Active

Two tests were carried out in each case except with amyl alcohol where but one was used. The results with acetone are not in harmony with the work of Bezssonov (1) who reports the isolation from cabbage juice of a curative substance which may be recrystallized from absolute acetone or absolute alcohol. We carried out three experiments with pure absolute acetone as a solvent and found in each case that the acetone-soluble portion was inactive and the acetone-insoluble portion was curative. When, however, we added a small amount of alcohol to the acetone, both the acetone-soluble and the acetone-insoluble fractions were curative.

None of the solvents tested gave as pure and uniformly curative extracts as did absolute alcohol. The influence of certain precipitants was therefore studied in an effort to obtain a purer extract. The tests were carried out on aqueous solutions prepared from the absolute alcohol extract as previously described. The following results were obtained:

Lead acetate, neutral	Filtrate	Residue
Phosphotungstic acid	Curative.	
“ “ in 5 per	“	Inactive.
cent sulfuric acid.	Inactive	“
Silicotungstic acid	Curative	
Silver lactate	Slightly curative.	
Phenylhydrazine	Inactive.	Inactive.

The results with lead acetate are in agreement with the findings of Silva (2).

Qualitative and quantitative analyses of curative extracts were carried out to obtain an index of the purity which might be obtained. Of the sugars there were identified the osazones of *d*-fructose and rhamnose. A few analyses are given in Table I,

calculated on the basis of mg. per 100 cc. of the original juice. Nitrogen was determined by the Kjeldahl method, using 0.02 N solutions for titration. Sulfur was determined by the Parr bomb method.

The great reduction in both phosphorus and sulfur values on treatment with neutral lead acetate solution indicates the probability that these elements are not a part of the antiscorbutic vitamin complex. Zilva (3) also found his purest extracts had low phosphorus content.

Amino nitrogen was always present, usually in about half the quantity of the total nitrogen. A natural indicator was also found, which was yellow in acid and reddish brown in alkali. In the alkaline state it may have been present as a salt, since it was

TABLE I

	Total solids	Nitrogen	Phos- phorus	Sulfur.
	mg	mg	mg	mg
Alcohol extract, control		5 48	2 22	0 9
Charcoal filtrate	744	4 3	1 67	
Lead acetate filtrate.			0 14	0 08
Uranium acetate filtrate			2 47	
Phenylhydrazine "			2 48	0 6
Silicotungstic acid "			0 63	1 4

soluble in water and insoluble in ether; the acid product was soluble in both water and ether. The recent work of Bezssonov (1) describes an indicator which he considers as the quinone formed by oxidation of his antiscorbutic substance. His quinone is red in acid and yellow in alkali.

SUMMARY.

1. Data on the solubility of the antiscorbutic vitamin in some of the common solvents are given. Of those investigated, ethyl alcohol gives the purest extracts.

2. Neutral lead acetate removes the bulk of the phosphorus and sulfur from these extracts without impairing their curative value.

3. The presence of a natural indicator, yellow in acid and reddish

brown in alkali, has been shown. It is apparently different from the one described by Bezssonov.

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BLOOD CHLORIDE METHODS.

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The large number of chloride methods which have been devised in the past few years is in itself *prima facie* evidence that no one method has proved universally satisfactory. Several methods such as those of Austin and Van Slyke (1), of Myers and Short (2), and of Smith (3) are satisfactory if only the one analysis is to be made on the blood. They are little used because they do not fit in with the Folin-Wu system of blood analysis (4) and therefore become cumbersome. The procedure described by Whitehorn (5) is a modified Volhard titration method using the Folin-Wu filtrate. Despite its advantages in simplicity and use of a common blood filtrate, this method in our opinion is not in all respects ideal. Personal factors determine largely the results obtained. Choice of the correct end-point is difficult, as the end-point can be made to fade or remain, depending upon the degree of stirring. Whitehorn described two conditions which could cause fading of end-point: reaction of sulfocyanate on the AgCl precipitate, and concentration of acid present in the mixture. The concentration of acid under the conditions imposed is constant, and is approximately 25 per cent. This should permit the end-point to remain for at least 15 minutes, according to Whitehorn. A greater concentration than this will result in a more rapid fading of the end-point. It is therefore probable that the usual difficulty in arriving at a satisfactory end-point with this method is due to the stirring and consequent increase in surface area of the silver chloride precipitate. Whitehorn in his original article mentioned the reaction of sodium sulfocyanate with the AgCl precipitate. This difficulty he attempted to overcome by flocking out the precipitate which was accomplished by adding concentrated HNO₃ and allowing the mixture to stand 5 minutes before titration.

However, we believe that in order to be able to select the end-point giving the correct result one must devote much time to the analysis of known specimens. Although we have observed every precaution we have never been able to feel any great degree of confidence in results obtained by the Whitehorn method. We therefore sought a method which would combine the advantages of simplicity and rapidity with a high degree of accuracy, and which at

TABLE I
Recovery of Added Sodium Chloride.

Specimen	100 cc of material			NaCl added	100 cc of material						Percentage error † Method.		
	Chlorides found • Method				Expected recovery Method			Recovery Method					
	Austin-Van Slyke	Whitehorn	Proposed		Austin-Van Slyke	Whitehorn	Proposed	Austin-Van Slyke	Whitehorn	Proposed	Austin-Van Slyke	Whitehorn	Proposed
	mg	mg	mg		mg per 100 cc	mg	mg	mg	mg	mg	mg		
Pooled blood	498	483	503	200	698	683	703	688	676	703	-5	-3 5	0
“ “	508	485	512	200	708	685	712	710	670	715	+1	-7 5	+1 5
“ “	494	481	491	200	694	681	691	692	668	693	-1	-6 5	+1
0.5 per cent aqueous NaCl solution					500	500	500	499	483	498	-0 2	-3 4	-0 4

* As NaCl.

† The percentages are based upon the recovery of *added* NaCl. Since a method would be expected to give the same result in a duplicate determination, it is assumed that any discrepancy lies in the recovery of added material.

the same time would be safe in the hands of any well trained routine laboratory technician. Because of its great definiteness and accuracy, the iodometric titration principle of McLean and Van Slyke (6) has long held high favor with us. As a volumetric procedure depending upon an indicator, the sharpness and permanency of end-point leave nothing to the imagination. Recoveries of added chloride have always approached 100 per cent and results are always consistent, as duplicate determinations show

excellent agreement. The attempt was made, therefore, to adapt the McLean-Van Slyke procedure to the Folin-Wu tungstic acid filtrate. Comparative results of three procedures are shown in Table I. A detailed description of our procedure follows.

*Description of Method.*¹

Reagents.

Sodium Tungstate.—A 10 per cent solution.

Sulfuric Acid.—A 2/3 N solution.

Silver Nitrate.—M/29.25. Dissolve 5.812 mg. of AgNO_3 in 600 cc of water in a liter flask; add 250 cc. of HNO_3 (sp. gr. 1.42). Dilute to mark with water.

Potassium Iodide.—M/73.1. Dissolve 2.4 mg. of KI in 1 liter of water. Standardize by titrating against 5 cc. of the AgNO_3 solution to which have been added 5 cc. of water and 6 cc. of starch indicator. Adjust so that the amount required will be 12.65 cc. (12.5 cc. to precipitate the AgNO_3 and 0.15 cc. to develop a definite end-point).

*Starch Indicator.*²—(A) Dissolve 2.5 gm. of soluble starch in 100 cc. of boiling water. Cool and dilute to 150 cc. (This solution should be prepared fresh each week.) (B) Dissolve 466 gm. of crystalline sodium citrate and 20 gm. of sodium nitrite in about 800 cc. of water. Dilute to 1350 cc. Solutions A and B are mixed in necessary quantities before determining chlorides, in the proportion of 1 to 9 respectively.

¹ After the details of this method had been worked out and adopted our attention was called to a similar method described by Gettler (7) using the same reagents in slightly different concentrations. Apparently Gettler's method did not receive great publicity because it was mentioned incidentally in an article alluding to another subject.

² The original reagent described by Van Slyke was made by dissolving 2.5 gm of soluble starch in 500 cc of water, adding 466 gm of sodium citrate and 20 gm of sodium nitrite. This was then cooled and diluted to 1000 cc. In this relatively concentrated solution there is a tendency for the sodium citrate to crystallize out, especially in cool weather. We have, therefore, changed the proportions so that 6 cc. of our mixture of Solutions A and B contain the same quantities of the various chemicals as originally described by McLean and Van Slyke. The extra 2 cc. of water do not affect the final result. The starch and citrate-nitrite solutions are kept separate so that the starch may be always fresh.

Procedure.

To 1 volume of oxalated blood or plasma (at least 3 cc.) add 7 volumes of water and mix. Then add 1 volume of the 10 per cent sodium tungstate solution and 1 volume of the $\frac{2}{3} N H_2SO_4$. Shake. After the mixture has changed to a chocolate-brown color, filter. To 20 cc. of the filtrate in a 50 cc. centrifuge tube, add 20 cc. of water, 10 cc. of the $AgNO_3$ solution; mix by stirring and centrifuge for about 5 minutes in a high powered centrifuge. Decant through a small filter paper. 20 cc. of this filtrate are then titrated with the standardized potassium iodide solution, using 6 cc. of the starch indicator. The end-point is a permanent deep blue color. Calculation $(10.15 - \text{cc. of KI solution used}) \times 100 = \text{mg. of NaCl in 100 cc. of blood or plasma.}$

SUMMARY AND CONCLUSIONS.

The objections to several blood chloride methods are pointed out, with special reference to the method of Whitehorn. The end-point in the Whitehorn method is unsatisfactory, as it yields results which usually are too low. The authors' technique, an adaptation of the McLean-Van Slyke iodometric principle to the Folin-Wu tungstic acid filtrate, is described. This is believed to be preferable to the Volhard titration. Tabulated results of a comparison of the methods of Austin and Van Slyke, of Whitehorn, and the proposed method are given. The proposed method possesses the advantages of being short, simple, and accurate.

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KINETICS OF INVERTASE ACTION.*

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If, in the case of the hydrolysis of sucrose by invertase, the velocity of hydrolysis is plotted against the concentration of sucrose, a curve is obtained like Curve IV, shown in Fig. 1. This curve shows that the rate of hydrolysis increases less and less as the concentration of sucrose increases and that it finally reaches a maximum when the concentration of the substrate has reached about 5 per cent.

Michaelis and Menten (1) were among the first to offer an explanation for this peculiar relationship between the rate of hydrolysis and sucrose concentration. In their study of this relationship, they made up, for instance, a series of eight sucrose-invertase solutions, all alike except that the concentration of sucrose varied from 0.77 M in the most concentrated to 0.0077 M in the most dilute, and measured the velocities at the beginning of each hydrolysis, when practically no invert sugar has been formed to exert a retarding influence. The initial velocities which they obtained are given in Column 3, Table I. When these values are plotted against the logarithm of their respective sucrose concentrations (Column 2) then a curve is obtained like Curve I shown in Fig. 1. The shape of this curve is very similar to that of an ordinary dissociation-residue¹ curve like Curves II or III shown in Fig. 1.

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¹ In order to describe briefly what is meant by a dissociation-residue curve, take the case of a simple reaction like



Let one of the components, say A, be added to the system in consecutive portions and let it be assumed that the reaction attains equilibrium im-

The fact that the dissociation-residue curve, which is only a graphical representation of the mass law principle, is similar in shape to their experimental curve, representing the relationship

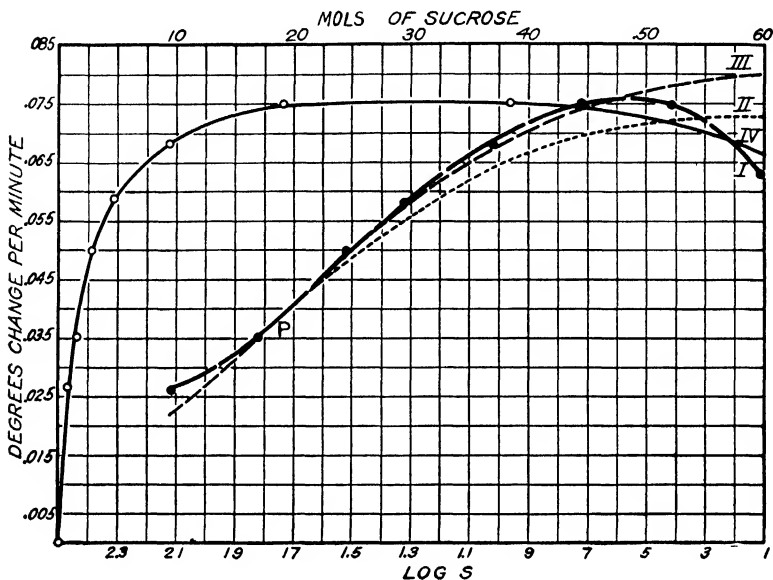


FIG 1

between velocity and sucrose concentration, led Michaelis and Menten to the idea that the invertase combines with the sucrose to form a compound, and that the velocity could be used as a

mediately after each addition of A. The relationship between the concentrations of the two components A and B and the compound AB can be expressed by

$$\frac{AB}{\text{Total B}} = \frac{A}{A + K} \quad (2)$$

in which K is the equilibrium constant. When the fraction of B existing in the combined state, *i.e.* $\frac{AB}{\text{total B}}$, is plotted against the logarithm of the concentration of uncombined A, then a curve is obtained like Curve II, given in Fig. 1. Since $\frac{AB}{\text{total B}}$ can be looked upon as the residue of the compound AB remaining in the undissociated form, this curve is sometimes known as a "dissociation-residue" curve.

relative measure of the concentration of this compound. Thus, if S , Θ , and φ represent the respective concentrations of free sucrose, total invertase, and sucrose-invertase compound, then

$$\frac{S \times (\Theta - \varphi)}{\varphi} = K \text{ or } \frac{\varphi}{\Theta} = \frac{S}{S + K} \quad (3)$$

It will be noticed that equation (3) has the same form as equation (2), having in place of $\frac{AB}{\text{total } B}$ the term, $\frac{\varphi}{\Theta}$, which represents the fraction of the total invertase existing as sucrose-invertase compound.

It is, however, impractical to determine, in any given sucrose-invertase solution, how much of the invertase is combined, and hence we cannot determine the value of the term, $\frac{\varphi}{\Theta}$, in equation (3). But, as already stated above, by assuming that the velocity is proportional to the fraction of the total invertase present as sucrose-invertase compound, $\frac{\varphi}{\Theta}$, equation (3) can be written

$$\frac{\varphi}{\Theta} = CV = \frac{S}{S + K} \quad (4)$$

The term CV is the velocity times a proportionality factor C , and the velocity can be measured. Concerning the concentration of free sucrose present in the solution, the quantity S in equation (4), Michaelis and Menten claim that the amount of invertase present is so small compared to that of the sucrose, that the amount of combined sucrose is negligible compared to the amount of sucrose used in making up the solutions, and since the velocities were measured at the beginning of the hydrolysis, the amount of free sucrose, S , can therefore be considered equal to the original sucrose concentration.

Equation (4) therefore represents the theoretical relationship, according to Michaelis and Menten's hypothesis, between the velocity of hydrolysis and the concentration of sucrose originally present in the solution. The proportionality factor C can be evaluated in either of two ways. The first method, which may be designated as the slope method, is as follows: A true dissociation-

residue curve has at its middle point, or its point of inflection, P in Fig. 1, a characteristic slope. If a straight line is drawn through this point tangent to the curve, then the tangent of the angle which this line makes with the abscissa axis will be equal to 0.576, provided the scale of units of the two coordinates is the same. By drawing a straight line tangent to the experimental curve at its point of inflection, which is not difficult to do, because, as can be seen, Curve I in Fig. 1 is practically a straight line for some distance, it is found that the slope is $\frac{0.063}{1.335} = 0.0472$.² This value 0.0472 is 12.206 times less than the characteristic slope 0.576. But it must be remembered that the ordinate units of the experimental curve are given in arbitrary velocity units, degrees change in rotation per minute. If it is assumed that the experimental curve is a dissociation-residue curve then, since the abscissa scale would be the same for the latter as for the experimental curve, *viz.* $\log S$, then the difference between the theoretical slope, 0.576, and the experimental slope, 0.0472, is due to the scale of arbitrary ordinate units being 12.206 times smaller than the units of the abscissa scale. Therefore, in order that the experimental curve shall have the same slope as a true dissociation-residue curve, it becomes necessary to stretch out, or multiply, the arbitrary velocity units by 12.206. Since the ordinates of the true dissociation-residue curve are $\frac{\varphi}{\Theta}$, and since $\frac{\varphi}{\Theta} = CV$, it follows that the factor, 12.206, is equal to the proportionality constant C in equation (4).

The second method, which can be called the maximum velocity method, for calculating the value of the constant C will now be described. As previously stated, the velocity of hydrolysis is supposed to be proportional to the concentration of the sucrose-invertase compound, and to the fraction of the invertase which is combined with sucrose, *viz.* $\frac{\varphi}{\Theta}$. Therefore, when the sucrose con-

² The values 0.063 and 1.335 are the respective values, in terms of the ordinates and abscissas, of the opposite side and base of a right angle triangle constructed with a portion of the tangent to the curve as the hypotenuse.

centration is such that the velocity is at its maximum, then $\frac{\varphi}{\Theta} = 1$.

In other words, from equation (4) we have

$$\frac{\varphi}{\Theta} = 1 = C \times (\text{maximum velocity}) \quad (5)$$

It will be seen in Table I, that the maximum velocity is about 0.075, and introducing this value in equation (5) we find $C = 13.333$. The reason why the values for C by the two methods are not exactly the same, is due, of course, to the fact that the experimental curve does not quite coincide with a true dissociation-residue curve.

TABLE I

S Initial concentra- tion of sucrose (1)	- log S (2)	V Experimental velocities, degrees per min (3)	Calculated velocities	
			C = 12.206 (4)	C = 13.333 (5)
0.7700	0.114	0.063	0.080	0.073
0.3850	0.414	0.075	0.078	0.072
0.1920	0.716	0.075	0.074	0.069
0.0960	1.017	0.0682	0.067	0.064
0.0480	1.318	0.0583	0.057	0.055
0.0308	1.517	0.0500	0.049	0.048
0.0154	1.813	0.0350	0.035	0.038
0.0077	2.114	0.0267	0.022	0.022

Calculation of Value of Equilibrium Constant, K, in Equation (4).—It can be seen, by inspection of equation (4), that when $\frac{\varphi}{\Theta}$ or $CV = \frac{1}{2}$, then S must equal K . Therefore, noting the point on the experimental curve, where the velocity is $\frac{1}{2}$ the maximum, the corresponding value on the abscissa axis will be the logarithm of the sucrose concentration which is equal to $\log K$. In the case of the maximum velocity being 0.075, then $\frac{1}{2}$ this value will be 0.0375, and from the experimental curve, in Fig. 1, it will be seen that $\log S = -1.76$ or $K = 0.0174$. When the slope of the experimental curve, at its point of inflection, is put equal to 0.576, then the theoretical maximum velocity, i.e. if the curve were a true dissociation-residue curve, will not be quite the same as the experimental value, because $CV = 1$ or 12.206 (see above) $\times V = 1$,

TABLE II.

Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min	Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)

Series A. Invertase K pH = 4.66 0.01 M citrate buffer.
Temperature 25°.

	<i>min</i>	<i>mg</i>			<i>min</i>	<i>mg</i>	
0.1 %	5	7.49		0.15 %	5	10.88	
2.53	10	13.37	1.40	2.36	10	18.87	2.05
	15	16.95			15	24.58	
0.25 %	5	16.25		0.5 %	5	27.64	
2.14	10	28.12	3.25	1.84	10	50.07	5.53
	15	38.07			15	67.71	
1.0 %	5	41.48		2.0 %	5	53.53	
1.53	10	78.15	8.29	1.23	10	101.68	10.7
	15	112.42			15	154.36	
3.0 %	5	61.3		4.0 %	5	65.3	
1.06	12	167.94	12.2	0.93	10	125.54	13.06
	15	183.64			15	189.95	
5.0 %	5	65.75		7.0 %	5	65.3	
0.84	10	127.49	13.15	0.69	10	128.30	13.06
	15	194.10			15	196.68	
10.0 %	5	64.1		15.0 %	5	63.43	
0.53	10	124.56	12.8	0.36	10	120.78	12.4
	15	189.28			15	178.36	

Series B Invertase 01AA pH = 7.3 0.01 M phosphate buffer
Temperature 25°.

0.15 %	5	2.92		0.5 %	5	4.92	
2.36	10	6.13	0.58	1.84	10	9.98	0.98
	15	9.06			15	15.12	
1.0 %	5	8.25		2.0 %	5	12.95	
1.53	10	15.25	1.65	1.23	10	22.81	2.40
	15	21.44			15	35.28	
4.0 %	5	16.04		5.0 %	5	15.94	
0.93	10	28.83	3.08	0.84	10	29.68	3.16
	15	41.40			15	45.90	
10.0 %	5	12.89					
0.53	10	24.96	2.56				
	15	37.15					

TABLE II—Continued

Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min	Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Series C Invertase 11 pH = 4.66 0.01 M citrate buffer.							
0.2 %	5	5.13		0.5 %	5	9.77	
2.23	10	9.81	1.02	1.84	10	19.80	1.95
	15	13.80			15	28.62	
1.0 %	5	14.75		2.0 %	5	19.93	
1.53	10	29.22	2.95	1.23	10	39.76	3.97
	15	42.92			15	60.53	
3.0 %	5	22.63		4.0 %	5	23.51	
1.06	10	44.81	4.49	0.93	10	47.05	4.70
	15	57.64			15	69.18	
5.0 %	5	24.20		10.0 %	5	22.45	
0.84	10	47.68	4.75	0.53	10	44.90	4.48
	15	71.12			15	68.12	
15.0 %	5	21.04					
0.36	10	42.09	4.2				
	15	64.50					
Series D Invertase 11 pH = 6.53 0.01 M phosphate buffer							
0.2 %	10	5.25		0.5 %	5	5.63	
2.23	20	10.35	0.52	1.84	10	12.22	1.15
	30	13.70			15	18.50	
2.0 %	5	14.38		3.0 %	10	31.80	
1.23	10	28.44	2.84	1.06	15	49.46	3.34
	20	55.20			20	67.24	
4.0 %	10	37.28		5.0 %	10	38.94	
0.93	15	55.72	3.73	0.84	15	59.39	3.86
	20	73.04			20	78.76	
7.0 %	10	38.44		10.0 %	10	0.23*	
0.69	15	57.28	3.84	0.53	15	0.35	3.59
	20	73.04			25	0.58	

TABLE II—*Continued*

Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min	Concentration of sucrose and $-\log S$	Time	Invert sugar	Initial velocity Mg invert sugar per min
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Series E Invertase 01AA pH = 4.66 0.01 M citrate buffer							
	<i>min</i>	<i>mg</i>			<i>min</i>	<i>mg</i>	
0.1 %	5	7.92		0.2 %	5	10.1	
2.53	10	14.80	1.55	2.23	10	19.06	2.0
	15	21.4			15	24.67	
0.5 %	5	26.02		1.0 %	5	37.60	
1.84	10	47.87	5.2	1.53	10	72.24	7.32
	15	64.95			15	104.24	
2.0 %	5	49.48		3.0 %	5	56.4	
1.23	10	99.20	9.9	1.06	10	112.6	11.2
	15	141.49			15	163.6	
4.0 %	5	61.00		5.0 %	5	63.22	
0.93	10	120.52	12.2	0.84	10	128.00	12.6
	15	174.94			15	191.36	
6.0 %	5	0.40*		7.0 %	10	0.79*	
0.76	10	0.80	12.5	0.69	20	1.58	12.3
	15	1.19			35	2.63	
10.0 %	10	0.77*		15.0 %	5	0.37	
0.53	25	1.81	11.96	0.36	10	0.74	11.50
	40	2.83			20	1.48	
Series F Invertase 01AA pH = 6.67 0.01 M citrate buffer.							
0.1 %	5	3.16		0.5 %	5	10.55	
2.53	10	6.14	0.63	1.84	10	20.86	2.10
	15	7.48			15	28.43	
1.0 %	5	18.68		2.0 %	5	26.72	
1.53	10	36.04	3.73	1.23	10	53.44	5.3
	15	54.72			15	79.16	
3.0 %	5	31.44		4.0 %	5	34.88	
1.06	10	60.80	6.2	0.93	10	69.08	6.98
	15	89.76			15	98.68	

TABLE II—*Concluded.*

Concentration of sucrose and $-\log S$	Time.	Invert sugar	Initial velocity Mg invert sugar per min.	Concentration of sucrose and $-\log S$	Time.	Invert sugar.	Initial velocity. Mg invert sugar per min
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Series F. Invertase 01AA. pH = 6.67 0.01 M citrate buffer— <i>Continued.</i>							
	<i>min</i>	<i>mg</i>			<i>min</i>	<i>mg</i>	
5.0 %	5	35.76		6.0 %	5	0.23*	
0.84	10	69.32	7.0	0.76	10	0.45	7.10
	15	102.40			15	0.65	
7.0 %	10	0.44*		10.0 %	10	0.42*	
0.69	20	0.86	6.86	0.53	15	0.63	6.51
	25	1.02			20	0.85	
15.0 %	5	0.20*					
0.36	10	0.40	6.24				
	20	0.79					

Constants C and K (equation (4)) for calculating theoretical curves.

Series	Curves II (maximum velocity method)		Curves III (slope method)	
	C	K	C	K
A	0.07575	0.019	0.06702	0.024
B	0.31645	0.027	0.24226	0.043
C	0.21008	0.020	0.17233	0.029
D	0.2577	0.029	0.19085	0.051
E	0.07936	0.021	0.07175	0.026
F	0.14084	0.028	0.10504	0.047

* Readings made by means of the polariscope.

and $V = 0.08192$. Half this maximum velocity will be 0.04096 and the abscissa value corresponding to this on the experimental curve is $\log S = -1.685$. The latter gives $K = 0.0206$.

Having determined the values for the two constants, C and K , in equation (4), it is now possible to calculate the theoretical velocities of hydrolysis, which should occur if Michaelis and Menten's theory is true, by substituting for S , in the equation, the values for S given in Column 1, Table I. The results from these calculations are given in Columns 4 and 5. The dotted lines in Fig. 1 were obtained by plotting the calculated velocities

against the corresponding sucrose concentrations. They are true dissociation-residue curves, and illustrate the deviation of the experimental results from Michaelis and Menten's theory.

The present authors have had occasion to carry out several series of hydrolyses, similar to the one described above. The procedure followed was slightly different, however, in that instead of using the polariscope for measuring the velocities, the copper reduction method described by Quisumbing and Thomas (2) was used. The reason for this departure was that only low sucrose concentrations (5 per cent and below) are significant as far as Michaelis and Menten's theory is concerned, and the copper method seemed to offer a greater degree of accuracy. The amount of cuprous oxide was determined by Bertrand's ferric sulfate-permanganate method.

All the hydrolyses were run at the same temperature, 25°, and, in each case, the concentrations of invertase and of hydrogen ion were kept the same. The initial velocities of hydrolyses, given in Table II, were obtained as follows. The amount of invert sugar formed was plotted against the corresponding time of the reaction and a straight line was then drawn tangent to the curve at its origin. By dividing the invert sugar value for any point on this line by the corresponding intercept on the time axis, the amount of invert sugar formed per minute at the beginning of the reaction was calculated. These initial velocities were then plotted, as ordinates, against the logarithms of the corresponding sucrose concentrations, as abscissas, just as described above in constructing the experimental Curve I in Fig. 1, from Michaelis and Menten's data, but due to lack of space they were not included in this paper. They can, however, be easily reproduced, if necessary, from the data given in Columns 1 and 4 in Table II. The constants, corresponding to $C = 12.206$ and $C = 13.333$ given in Table I and derived in the same way, used for calculating the theoretical velocities by either the maximum velocity or slope methods, are given at the bottom of Table II. The curves obtained from the data in Table II agreed quite well with the shape of the Michaelis and Menten curve (Curve I, Fig. 1). They were practically straight lines up to about 5 per cent sucrose ($\log S = -0.84$) at which point the maximum velocity occurred. They differed from the Michaelis and Menten curve, however,

in having a more restricted region of constant maximum velocity, there being usually a perceptible decrease in velocity even when the sucrose concentration reached 7 per cent. Hence, in this respect, their shapes resembled more the maximum portion of the curve shown in Fig. 2. There was, in each case, quite a noticeable deviation from the corresponding theoretical curve, based upon Michaelis and Menten's theory, whether the latter was constructed by the maximum velocity method (like Curve II, Fig. 1) or by the slope method (like Curve III). Michaelis (3) in defending his hypothesis points out that it is well known that the mass law only holds strictly for very dilute solutions, and it will be noted that the deviation appears to be the greatest in the region of the higher sucrose concentrations. Nevertheless, the justification for the theory rests upon the agreement between the experimental and the theoretical dissociation-residue curves.

It is quite generally accepted that invertase is colloidal, at least in the form of the preparations as used up to the present (4). Bayliss (5) states:

"We may suppose that, in hydrolysis, for example, the substrate is brought into intimate relation with water on the surface of the particles of the enzyme . . . It must be understood, that adsorption is merely a preliminary stage, and that after it has taken place, the proper chemical reaction makes its appearance. The rate at which the reaction proceeds is controlled by mass-action on the part of the adsorbed substances and is, therefore, in proportion to the amount adsorbed."

Michaelis (6) recognizes the fact that invertase preparations show many properties which indicate a colloidal nature, but cites an array of experimental evidence which, he claims, shows that as far as the action of the enzyme on sucrose is concerned, this reaction is not contradictory to requirements of the mass law. Willstatter and Kuhn (7), who have adopted Michaelis' views in the interpretation of much of their experimental data, also advance several arguments similar in character to those of Michaelis.

In conclusion it might be said, that whether or not the hydrolysis of sucrose by invertase should be considered as heterogeneous or homogeneous is still an open question. This is due chiefly to the fact, as Josephson (8) has recently shown, that the actual amount of invertase, *i.e.* active principle, present in a sucrose

solution undergoing hydrolysis at such a rate as to be followed by accurate measurements, is negligible compared to the concentration of the sucrose. If we accept the assumption that the velocity is proportional to the sucrose-invertase compound, and since we know that doubling the concentration of enzyme doubles, within experimental error, the velocity, it follows that whether equation (3) is written

$$\frac{S \times (\theta - \varphi)}{\varphi} = K \quad \text{or} \quad \frac{S \times (2\theta - 2\varphi)}{2\varphi} = K$$

makes no difference as far as the mass law is concerned, because the 2 cancels out in the last equation.

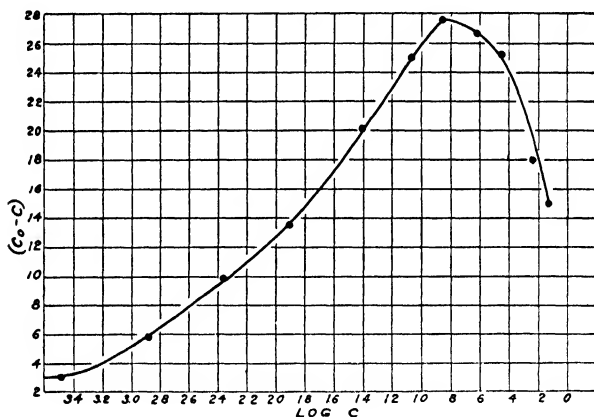


FIG. 2.

Nelson and Vosburgh (9) considered the relationship between velocity of hydrolysis and sucrose concentration from the standpoint of a heterogeneous reaction. They pointed out that according to Schmidt (10) the adsorption of acetic acid in water to charcoal reached a maximum value as the concentration of the acid was increased, and if it is assumed that the velocity of hydrolysis is a measure of the sucrose adsorbed to the invertase, then the two phenomena appear to be very much alike. Since then Dora Schmidt-Walter (11) has also made some measurements of the adsorption of acetic acid, from aqueous solutions, by charcoal. She has calculated from her experimental data the gm. of acetic

acid, in Fig. 2, adsorbed from each of a series of 100 gm. solutions, of varying acid content, by 10 gm. of charcoal. In Fig. 2, these calculated gm. of acetic acid adsorbed have been plotted as ordinates against the logarithm of the per cent by weight of acetic acid remaining in the solution after equilibrium was attained. It will be noticed that the amount of acid adsorbed reaches a maximum and then decreases as the concentration of the acid in the solution increases still more. The shape of this curve bears a striking resemblance to the experimental velocity-logarithm sucrose concentration curves obtained from the data given in Table II. Williams (12), Schmidt-Walter, and others attribute this decrease in the amount of acetic acid removed by the charcoal, from 100 gm. of the solution in the higher concentrations, to the solvent, in this case water, being adsorbed also.

Since the relationship, between the relative amounts of acetic acid adsorbed to charcoal and the logarithm of the concentration of the acid, gives a curve which is very similar in shape to the curve obtained by plotting the velocity of hydrolysis against the logarithm of the sucrose concentration, it follows that here is another curve, besides the dissociation-residue curve, which can also be taken to indicate how the invertase combines with the sucrose. If, eventually, it should prove to be nearer the truth than the simple mass law idea, then it will be necessary to take into consideration also the rôle played by water, instead of just the sucrose as Michaelis and Menten have done.

The suggestion that the decrease in velocity of hydrolysis of the sucrose at higher concentrations of the latter is not due so much to the mass law breaking down in the concentrated solutions, as claimed by Michaelis (3) and von Euler and Myrbeck (13), but rather to the two reactants, sucrose and water, both combining with the enzyme, gains support from a statement made by Freundlich (14) in discussing catalytic reactions in which two reactants are involved.

"In this group of processes the velocity will certainly depend upon the concentration of the two adsorbed materials at the boundary surface. Now since one substance displaces the other, we have the important conclusion that the velocity, as it varies with the concentration (or amount adsorbed as the case may be) of each of the substances, passes through a maximum "

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STIMULATION OF THE SUGAR-REGULATING MECHANISM AS SHOWN BY DUPLICATE BLOOD SUGAR CURVES.*

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After the oral administration of glucose, when the concentration of the sugar in the blood is falling, ingestion of a second dose of glucose may result in little or no increase in blood sugar. This occurred in nine out of thirteen experiments conducted by Foster (1). Maclean and de Wesselow (2) had previously reported a single case in which a second dose of glucose produced a curve which was nearly as high as the first. Foster used 100 gm. and Maclean only 50. As we shall show later, this difference in the amounts of glucose used may account for the discrepancy in the results of the two observers. One or two observations have been reported also by Staub (3), Traugott (4), and du Vigneaud and Karr (5). As an explanation of this lack of hyperglycemia after a second dose of glucose, Foster accepts the suggestion proposed by Frank (6) and others for the hypoglycemia following the administration of glucose; *viz.*, that the glucose overstimulates the formation of glycogen. Foster's observations disprove the explanation of Folin and Berglund (7), that such hypoglycemia is due to decreased need for transport of glucose to the tissues.

In a study of the blood sugar curves of a group of 170 non-diabetic individuals, we encountered many bizarre results. It occurred to us that additional information concerning the carbohydrate metabolism of some of these subjects might be obtained

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by comparing their response to duplicate doses of glucose when administered both by mouth and intravenously. We wished to observe the response to various amounts of glucose and to determine whether ingested or intravenously injected glucose had the greater effect in stimulating the process which causes reduction in the concentration of sugar in the blood.

Material and Methods.

Most of our subjects had had previous repeated blood sugar curve tests. They were therefore accustomed to the procedure, and we were familiar with their type of response. Twenty-four subjects were used for these experiments. Two were healthy persons, the others were patients subject to recurring convulsions. For the ingestion experiments, we used 1.5, 0.75, and 0.33 gm. of glucose per kilo of body weight in 33 per cent solution. For the injection experiments, we used 1.5 and 0.33 gm. of glucose per kilo of body weight in 20 per cent watery solution. In all experiments the duplicate dose of glucose was of the same amount as the first. The interval between doses varied from 20 minutes to 2 hours. Venous blood was drawn at frequent intervals, as noted in the accompanying charts. Blood sugar was measured by the method of Folin and Wu (8), using the sugar tubes suggested by Rothberg and Evans (9). Further details concerning material and methods are given in other publications (10-12). For the sake of brevity we shall speak of curves following ingestion and intravenous injection of glucose as ingestion and injection curves respectively. The terms first and second curves refer to blood sugar curves following the initial and the duplicate administration of glucose in the same experiment.

Results.

Of the twenty-four subjects used, twenty had tests performed both by ingestion and intravenous injection. In all, 56 duplicate curves were made, of which thirty were double ingestion and twenty-six were double injection curves. The blood sugar curves obtained in these experiments were so variable that it is difficult to display results by means of illustrative cases. We have, however, presented data of twenty-two of the 56 experiments in Figs.

1 to 5 which follow. In each of the first four charts the upper dotted lines represent injection and the lower solid lines ingestion curves. The solid squares and rectangles indicate time when glucose was given and the approximate relative amounts. In the injection experiments shown in the first four figures 0.33 gm. per kilo of glucose was used.

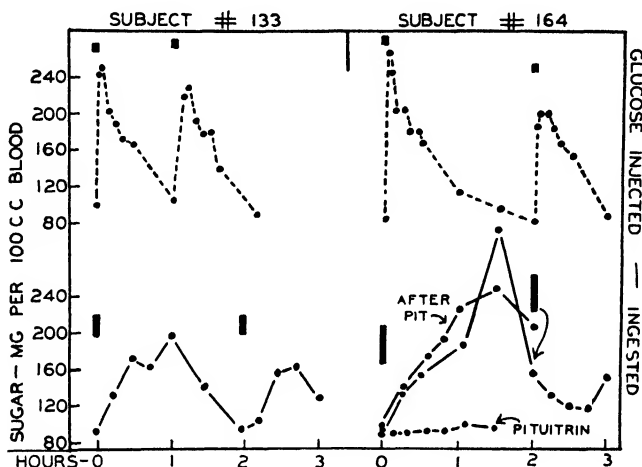


FIG 1 Blood sugar curves of Subjects 133 and 164. The latter was a patient with diabetes insipidus. Double injection and ingestion curves were made before institution of treatment. Ingestion curve marked "after pit" was begun 10 minutes after intramuscular injection of 1 cc of pituitrin. Curve marked "pituitrin" was made following injection of 1 cc. of pituitrin. In this experiment no glucose was administered. The abscissa represents minutes and the ordinate measures mg of sugar per 100 cc of whole blood. In this and the three subsequent figures the broken lines indicate intravenous blood sugar curves and solid lines ingestion curves. Solid squares and rectangles indicate time when glucose was administered and the approximate relative amounts. The amounts of glucose used were as follows: for ingestion, 1.5, 0.75, and 0.33 gm per kilo of body weight, and for injection, 1.5 and 0.33 gm per kilo of body weight.

The second half of Fig. 1 details four experiments with Subject 164, a patient with diabetes insipidus. The second injection curve of this subject was somewhat lower than the first. An hour and a half after the ingestion of 100 gm. of glucose, blood sugar had risen to a height of 315 mg. Ingestion of a second 100 gm. of

glucose at the end of 2 hours did not prevent the continued fall of blood sugar. On another occasion, a blood sugar curve test was begun 10 minutes after intramuscular injection of 1 cc. of pituitrin. The effect of the pituitrin was reflected in an excretion of only 370 cc. of urine against an output of 1470 cc. during the corresponding 2 hour period of the previous test. In spite of this fact, however, the blood sugar curve (marked "after pit." in the chart) was similar to the previous curve. On another occasion injection of 1 cc. of pituitrin intramuscularly resulted in no increase

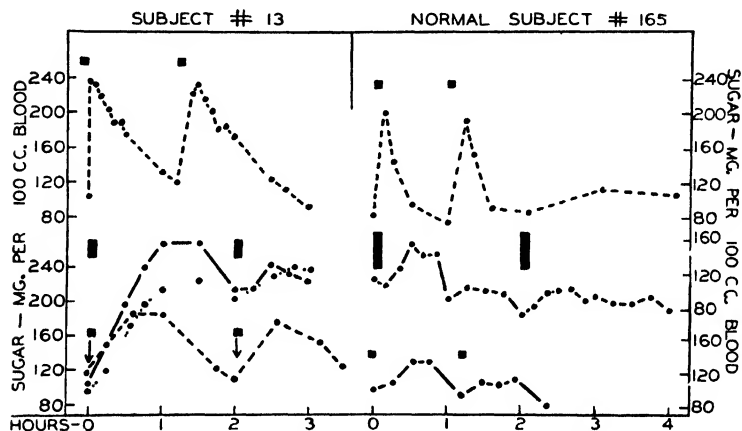


FIG 2 One duplicate injection and three duplicate ingestion curves of Subject 13. Ingestion curves which are marked by solid and dotted lines followed the ingestion of 0.75 gm. of glucose per kilo. The lowest curve with broken lines followed ingestion of 0.33 gm. per kilo. The upper ingestion curve of normal Subject 165 followed ingestion of 1.5 gm. and the lower curve ingestion of 0.33 gm. of glucose per kilo.

in the level of sugar in the blood. In this individual, therefore, the alimentary hyperglycemia which he showed did not seem to be influenced by the injection of pituitrin.

The first half of Fig. 1 illustrates an experiment in which a double dose of 0.75 gm. of glucose was ingested. In this instance there was a rise of blood sugar following the second ingestion. In other instances in which the subjects drank the second solution of glucose while blood sugar was yet elevated, the concentration of sugar in the blood continued to fall.

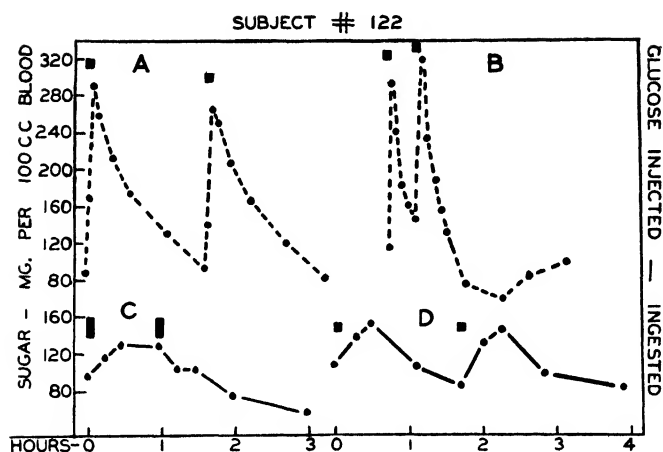


FIG. 3 Two duplicate injection and ingestion curves of Subject 122. In the experiment marked A the interval between injections was 90 minutes. In that marked B the interval was 20 minutes. In Experiment C 0.75 gm. of glucose per kilo was ingested, and in Experiment D 0.33 gm. per kilo.

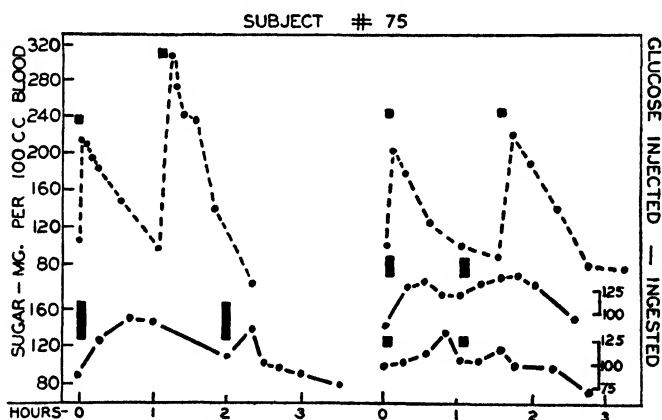


FIG. 4. Two duplicate injection and three duplicate ingestion curves of Subject 75. In the ingestion experiments the amounts of glucose used were 1.5, 0.75, and 0.33 gm. per kilo. Other injection curves of this subject are shown in Fig. 5.

The response to the second dose of glucose differed so greatly in different individuals that we wished to determine the extent of

the variation at different times in the same individual. In making the eighteen double curves shown in Figs. 2 to 5 four subjects were used. Subject 13 (Fig. 2) was a patient whose ingestion curve was constantly diabetic in type. On two occasions, double

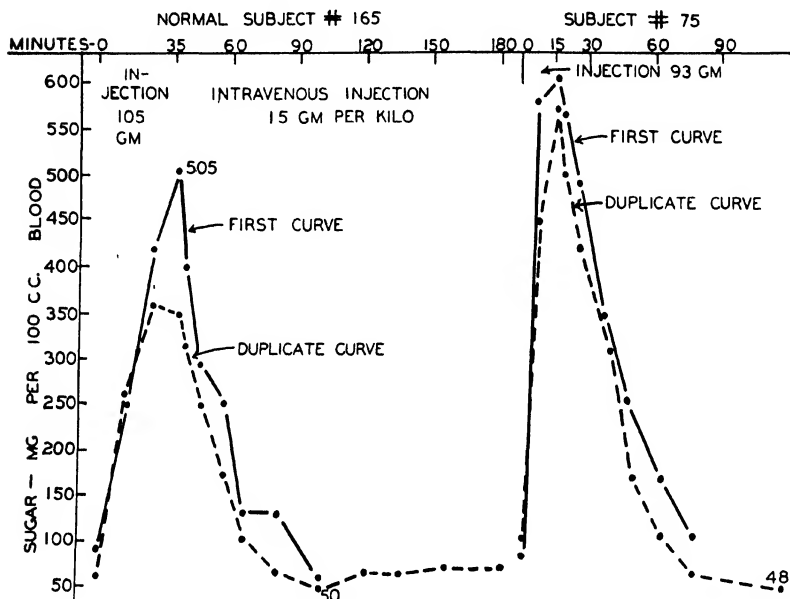


FIG 5 Duplicate curves following intravenous injection of 1.5 gm of glucose per kilo of body weight in normal Subject 165 and in Patient 75. The dotted zones indicate the period during which injections were made. In the case of Subject 165, injection was at the rate of 3 gm of glucose per minute, 35 minutes being required. In Subject 75, injection was at double this rate; namely, 6 gm per minute, the time required being approximately 15 minutes. In this chart solid lines indicate initial curves and broken lines duplicate curves. The two are superimposed. During the period of injection blood was drawn from the arm not being used for injection. In both subjects second injection of glucose resulted in marked degree of hypoglycemia, 48 and 50 mg per 100 cc, respectively.

ingestion curves were made using 0.75 gm. of glucose per kilo. The resulting curves were approximately the same. Fig. 3 shows four experiments on one subject. When sugar was injected after an interval of $1\frac{1}{2}$ hours (Curve A), the second curve was

slightly lower than the first. When the interval between the two injections was only 20 minutes (Curve B), the second curve, except for the measurement at the end of injection, was again slightly lower than the first. When a second 0.75 gm. per kilo of glucose was ingested (Curve C), there was no consequent rise in blood sugar. When only 0.33 gm. per kilo was used (Curve D), the second rise was greater than the first. Fig. 4 presents five double sugar curves of Subject 75. In both instances in which glucose was injected, the second injection curve was higher than the first. Varying results were obtained when different amounts of glucose were ingested.

Inspection of the foregoing figures makes it evident that *with relation to initial curves* second intravenous curves were higher than second ingestion curves. This may be due to the fact that only small amounts of glucose were injected. Fig. 5 presents two experiments in which duplicate injections of 1.5 gm. of glucose per kilo were made. Previous double injection curves of these subjects, with use of 0.33 gm. per kilo of glucose, are shown in Figs. 2 and 4. In two injection experiments of Subject 75 (Fig. 4), second curves had been considerably larger in area than first curves. In the experiments detailed in Fig. 5, more than 4 times the previous amounts of glucose were injected. In both experiments second curves were lower than initial curves. Another point of difference between large and small injections of glucose was the more marked hypoglycemia which followed the use of the larger amount. After the double injection of 0.33 gm. of glucose per kilo, the lowest concentration of blood sugar was 87 mg. for Subject 165 and 74 mg. for Subject 75. After the double injection of 1.5 gm. of glucose, the corresponding values for these subjects were 50 and 48 mg. In each subject, if all the sugar had remained in the blood during the period of injection, blood sugar at the end of injection would have been approximately 1700 mg. per 100 cc. of blood. Instead, blood sugar of Subject 75 was 606 mg. and of Subject 165, 505 mg. In other words 64 per cent of the injected glucose disappeared from the blood during the 15 minute injection of Subject 75, and 70 per cent during the 35 minute injection of Subject 165. During second injections, 66 per cent of the injected glucose disappeared from the blood of Subject 75, and 80 per cent from the blood of Subject 165. Therefore, 8 per cent more of

glucose disappeared from the blood when glucose was injected more slowly. During the last 11 minutes of the second injection of Subject 165, when glucose was being introduced at the rate of 3 gm. a minute, the concentration of the sugar remained constant. During the 11 minute period after the injection was finished, glucose disappeared from the blood at the rate of approximately 0.5 gm. a minute. The impetus which the sugar-disposing mechanism received by this double injection of glucose is shown most strikingly in Subject 75. During the 65 minute period after the second injection, concentration of blood sugar decreased 500 mg., a rate of nearly 8 mg per 100 cc a minute. In a diabetic person, it would require the injection of large amounts of insulin to produce such a rapid and extensive fall in the concentration of blood sugar. During the period when blood sugar was approximately 50 mg. per 100 cc., Subject 165 experienced symptoms of a mild hypoglycemic reaction.

In order to make comparison of these double curves, it is necessary to express the degree of hyperglycemia which follows administration of glucose by a number. Because of the variability in the form of curves and also because of the different intervals at which blood was drawn, the use of a formula is not feasible. Therefore, we have expressed the height of curves in terms of the area covered. To do this, we drew curves on cross-ruled paper, transferred them to tracing cloth which weighed approximately 0.1 mg per sq. mm. of surface, cut out the areas which were above or below the base line formed by the fasting blood sugar, and weighed. Areas below the fasting base line were subtracted from those above.

Areas of second injection curves were from 280 to 29 per cent of the areas of the first injection curves. Of the twenty-six double injection curves, areas of second curves were greater than areas of first curves in twelve experiments and smaller in fourteen. Second ingestion curves showed an even greater variation. Of the thirty ingestion experiments, areas of second curves were greater than areas of first curves in six experiments and smaller in twenty-four. In nine instances second curves had a negative value; *i.e.*, after the ingestion of glucose blood sugar for the most part was below the preingestion level. For individuals, there was no constant relationship between the relative areas of double ingestion and in-

jection curves. A subject with a relatively high second injection curve might have a relatively low second ingestion curve and *vice versa*.

Certain data are summarized in Table I. Comparison of the first two lines of Table I shows that increase in blood sugar following a second dose of glucose was much smaller if blood sugar was still elevated when the second dose was given. Apparently there was increased speed in the disposal of glucose when blood sugar was elevated. This is seen also by the shape of intravenous curves; *e.g.*, those shown in Fig. 5. The rate of removal of sugar from the blood was greatest during the latter part of the period of injection

TABLE I
Summary of the Average Relative Areas of Various Curves

Glucose	Blood sugar at beginning of 2nd curve	Glucose administered per kilo							
		1.5 gm		0.75 gm		0.33 gm		All cases	
		No of cases	2nd curve, percent of 1st	No of cases	2nd curve, percent of 1st	No of cases	2nd curve, percent of 1st	No of cases	2nd curve, percent of 1st
Ingested	Normal	7	54	7	75	3	116	17	73
	Elevated	4	4	8	45	1	5	13	28
	Both groups	11	28	15	53	4	101	30	47
Injected	Normal	2	75			24	104	26	96
All experiments.		13	52	15	53	28	104	56	78

and immediately after, when hyperglycemia was greatest. After injection the curves gradually flattened out into a straight line.

A general summary of the relative areas of the 56 duplicate blood sugar curves is presented in Fig. 6. Inspection of this figure shows the following: First, the amount of glucose being equal, the area of a blood sugar curve was much greater following intravenous injection than following oral administration of the glucose. This was especially true when large amounts of glucose were used. In Fig. 6 the area of the first double column (representing the curves following injection of 1.5 gm. per kilo of glucose) is more than 8 times the area of the second double column (representing curves following ingestion of the same amount of glucose). Second, with

both methods of administration the area of the second curve with relation to the first was much smaller when larger amounts of glucose were used. This is seen most clearly in the heights of the columns at the bottom of the figure. Following the oral administration of 1.5 gm. per kilo, the second curve was ap-

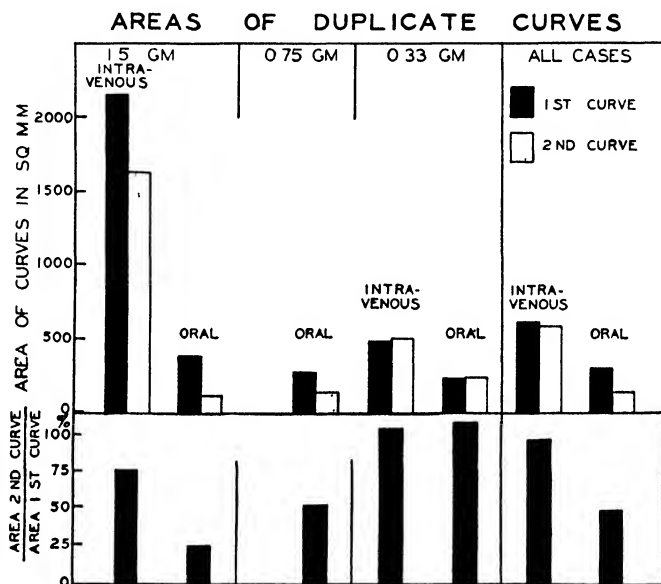


FIG 6 Graphic representation of the relative areas of first and second injection and ingestion curves following administration of various amounts of glucose. In the upper part of the chart the ordinate represents the area of the curves expressed in sq mm. The method used in measuring the area is explained in the text. In the lower part of the chart the ordinate represents the area of the second curve with reference to the area of the first. The measurements are grouped into the experiments in which 1.5, 0.75, and 0.33 gm per kilo of glucose were used. In the upper half of the chart areas of initial curves are represented by solid columns and of duplicate curves by dotted columns.

proximately one-fourth the area of the first. With 0.75 gm., it was one-half the area of the first, and with 0.33 gm., it was of approximately the same area. In other words, the relative area of second curves, roughly speaking, was inversely proportional to the amount of glucose ingested. The contrast in the response to

large and small doses was not so marked when glucose was injected. It would seem clear that small amounts of glucose do not over-stimulate the sugar-regulating mechanism to the same degree as large amounts. Third, with large amounts of glucose the sugar-disposing mechanism was stimulated more by ingestion than by injection of glucose. Following intravenous injection of 1.5 gm. per kilo (two experiments) the second curve was three-fourths the area of the first, whereas where glucose was ingested the second curve was but 28 per cent of the first (first two columns at the bottom of Fig. 6). If we exclude the four experiments in which the second dose of glucose was ingested when blood sugar was elevated, the proportion is 54 per cent.

There are various possible explanations of this fact that a second dose of glucose when given by mouth does not cause as great an increase of blood sugar as when injected intravenously. First, in contrast with injected glucose, the second dose of ingested glucose may not enter the blood as rapidly as the initial dose. We have no means of knowing how important this factor is. Second, the fact that ingested glucose enters the blood stream more slowly than we injected it may influence the height of curves. Holm (13) has reported that a given dose of insulin has a more marked effect when injected over a period of hours than when injected in one dose. As we have already pointed out, in the experiments shown in Fig. 5, when sugar was injected at a slow rate, at the end of injection 8 per cent more sugar had disappeared from the blood than when sugar was injected at a faster rate. However, if the total areas of the second curves, instead of the height of glycemia, are considered, we find that the experiment in which glucose was injected more slowly showed the larger area for the second curve. Our observations are too few to permit conclusions on this point. Third, it is possible that glucose which is injected directly into the blood stream causes less stimulation of the blood sugar-regulating mechanism than glucose which passes first through the intestines and liver. The experiments of Franke and Wagner (14) in which they find that after peritoneal injections of glucose the fermentable sugar is transformed into higher non-fermentable carbohydrates only after a certain period of time, suggest this. It is evident that the sugar-regulating mechanism receives greater stimulus from ingested than from injected glucose, but we have no means of judging which of the factors named is most concerned.

Our experiments confirm the importance of glucose as a stimulator of the blood sugar-regulating mechanism of the body. Elsewhere (11) we shall present observations concerning the alimentary hyperglycemia which occurs in fasting, a condition which apparently is due to lack of stimulation of the sugar-regulating mechanism. Maclean and de Wesselow (2) thought that such stimulation became effective only when concentration of sugar in the blood exceeded the renal threshold. Although, as we have seen, the process of removal of sugar from the blood was greatly increased when glycemia was great, in many of the ingestion experiments this process was hastened when there was but slight increase in blood sugar. Our experiments would seem to demonstrate that the degree of stimulation of the sugar-disposing mechanism is dependent not on the height of blood sugar *per se*, but rather on the amount of glucose introduced into the body. Our experiments do not permit analysis of the possible factors concerned in the increased rapidity with which sugar is removed from the blood. Such factors are an increased rate of oxidation of glucose or of its absorption into the tissues or an increased rate of glycogen formation. Foster (1) believes the last named factor is the one of most importance.

The foregoing observations are concerned only with non-diabetic subjects. In such subjects preliminary administration of 0.33 gm. per kilo—about 20 gm. in all—of glucose seems to have little influence in hastening removal from the blood of a subsequent similar dose of glucose. The behavior in diabetic patients would seem to deserve some study. It is possible that patients could utilize carbohydrate food better if it were given in small amounts frequently repeated.

SUMMARY.

We have performed 56 experiments in which duplicate amounts of glucose were administered at intervals of from 20 minutes to 2 hours. In twenty-six of the experiments glucose was given intravenously, and in thirty it was given by mouth.

1. The response of various subjects, and of the same subjects to the two methods of administration, differed greatly.

2. Following ingestion of various amounts of glucose, the average area of the second curves was inversely proportional to the amount of glucose ingested.

3. Areas of curves were much larger following injection than following ingestion of glucose. Also, areas of second duplicate curves (in relation to initial curves) were larger when glucose was injected than when it was ingested. Following double injection of glucose, hypoglycemia as low as 48 mg. per 100 cc. of blood was reached.

4. These observations emphasize the importance of glucose as a stimulator of the blood sugar-regulating mechanism of the body.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM. IV.

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A comparative study of the respiratory function of the blood may be made with the help of the methods developed in the preceding papers of this series (1-3). Such a study, because founded upon a mathematical description, possesses certain advantages which are uncommon in comparative biological researches. For example, it is possible to compare the differences in composition and in respiratory exchange of blood, at rest and at work, in normal and in pathological states, in man and in other animals. Thus, in the end, all the obviously important biological variables may be simultaneously considered and the correlative physicochemical variations may be noted and interpreted.

In the present paper the results of the study of the blood of a second normal man (C.V.C.) at rest are reported, certain slight modifications in the nomographic representation of the results are introduced as a means to facilitate comparison, and complete nomograms for the two men, C.V.C. and A.V.B., and for the horse studied by Van Slyke, Wu, and McLean (4) are presented.

The first step in the investigation consisted in measurements of equilibrium at 37.5° between oxygen, carbon dioxide, and the blood of C.V.C., in order to construct oxygen dissociation curves and carbon dioxide dissociation curves of whole blood and curves representing the composition of true plasma. The methods employed were those described in the paper of Bock, Field, and Adair (5). The data are represented in Figs. 1 to 3.

The volume of cells, expressed as per cent of the volume of the

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blood, was estimated with the help of the hematocrit and by the oxygen capacity. These data and a few observations on water and chlorides are given in Table I.

A few observations were also made upon the refractive index of true plasma. These are collected in Table II.

TABLE I

Date	Cell volume	Oxygen capacity	Serum NaCl	Serum H ₂ O	R Q	Alveolar pCO ₂	Arterial pCO ₂
1928	per cent	vol per cent	mg per 100 cc serum	cc per 100 cc serum		mm	mm
Nov. 16		19 93	598	92 2	0 85	41 4	
" 18		20 50			0 88	42 5	
" 24	42 0	19 92	593		0 84	42 0	
" 27*	41 0	18 15			0 83	42 2	41 4
" 29					0 75	44 0	
Dec 21†		20 00			0 90	37 6	

* The blood drawn on Nov. 27 was arterial. The other specimens were venous.

† In this experiment the subject was standing. In the other experiments the subject was reclining and in a postabsorptive condition.

TABLE II.

Index of Refraction of True Plasma.

Date	pCO ₂	pO ₂	[n] _D serum - [n] _D water
1928	mm	mm	
Nov. 16	21 8	Air.	0 01477
	39 2	"	0 01481
	95 4	"	0 01508
	16 1	2 1	0 01496
	32 2	2 6	0 01507
	87 2	2 3	0 01560
Dec. 20	55 4	Air.	0 01560

From the above data, with the help of the equations of Van Slyke, Wu, and McLean (4) and the more recent investigations of Van Slyke and his collaborators (6), the complete nomogram, Fig. 4, was constructed. The construction corresponds in all respects to that described in the second paper of this series (2).

After slight and unimportant modifications, in order to adjust

the value of pH_c and the values of r to the more recent estimates of the true nature of these functions, in accordance with the values employed in the construction of Fig. 4, the nomogram of the blood of A.V.B. (2) was reduced to the scale of Fig. 4 and is

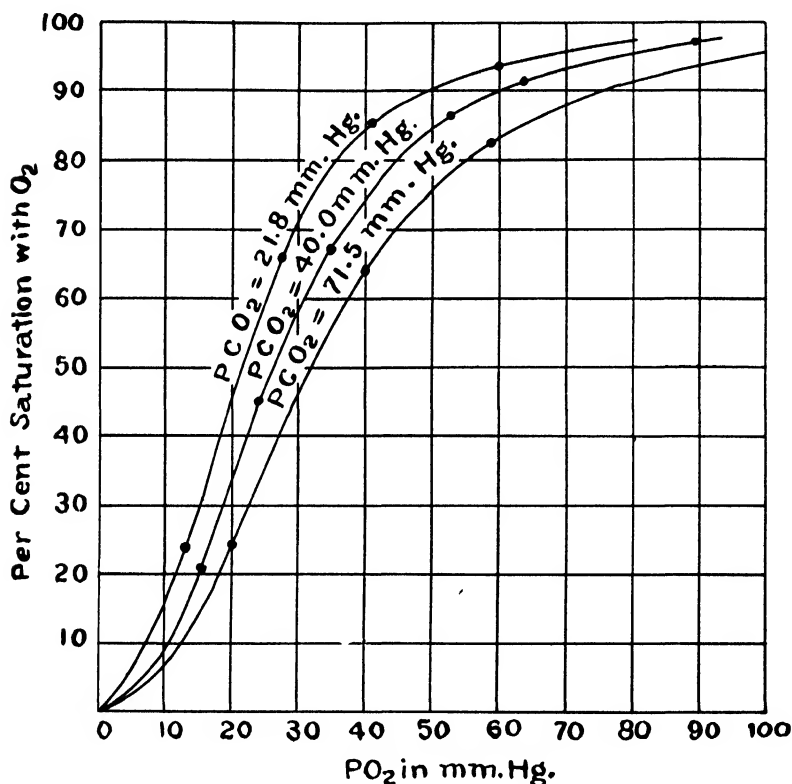


FIG. 1. O₂ dissociation curves.

printed as Fig. 5. A similar transformation of Van Slyke, Wu, and McLean's nomogram of horse blood (4) is given in Fig. 6.

Tables III to V present the composition of whole blood, of plasma, and of cells during rest, in arterial blood and in mixed venous blood, and also the respiratory exchanges as defined by the two nomograms. It should be particularly noted that the venous

line of the *original* nomogram of the blood of A.V.B. corresponds to a condition of moderate activity (oxygen consumption about 500 cc. per minute, or about twice that here considered). For the horse we have arbitrarily assumed a coefficient of utilization of oxygen of the same order as that observed in man, so as to make possible a comparison of his blood with human blood functioning through the same cycle.

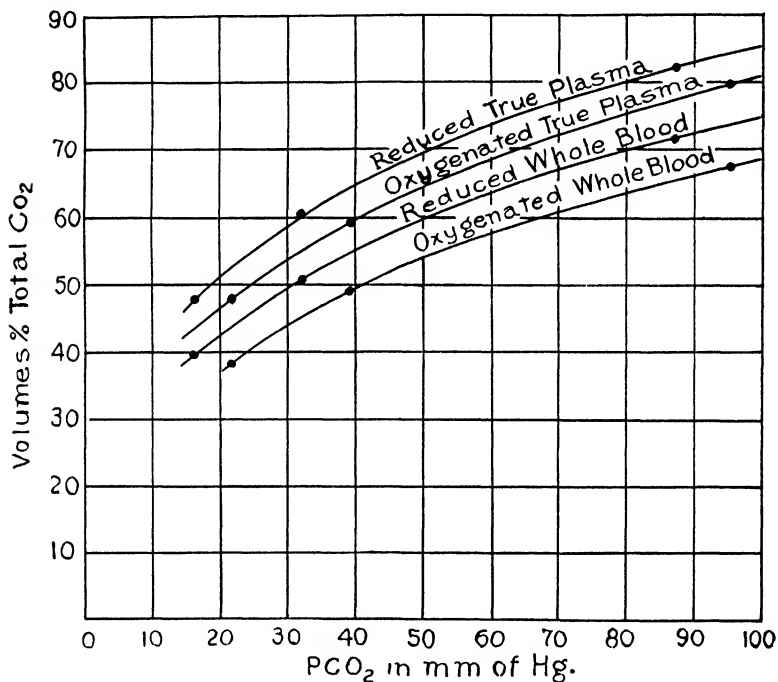


FIG. 2. CO₂ dissociation curves.

The two human bloods are nearly identical in all respects. Slight differences in carbonic acid capacity exist, and these are accompanied by trivial dependent variations in other variables. From time to time in both of these individuals there are also fluctuations in oxygen capacity, and it is a matter of chance that the two bloods are found to be so closely allied in this respect on the days chosen for study. The measurements reported in this

paper and others not yet published seem to indicate that normal human blood under "basic" conditions is of nearly constant composition except for variations (1) in the proportion of total base

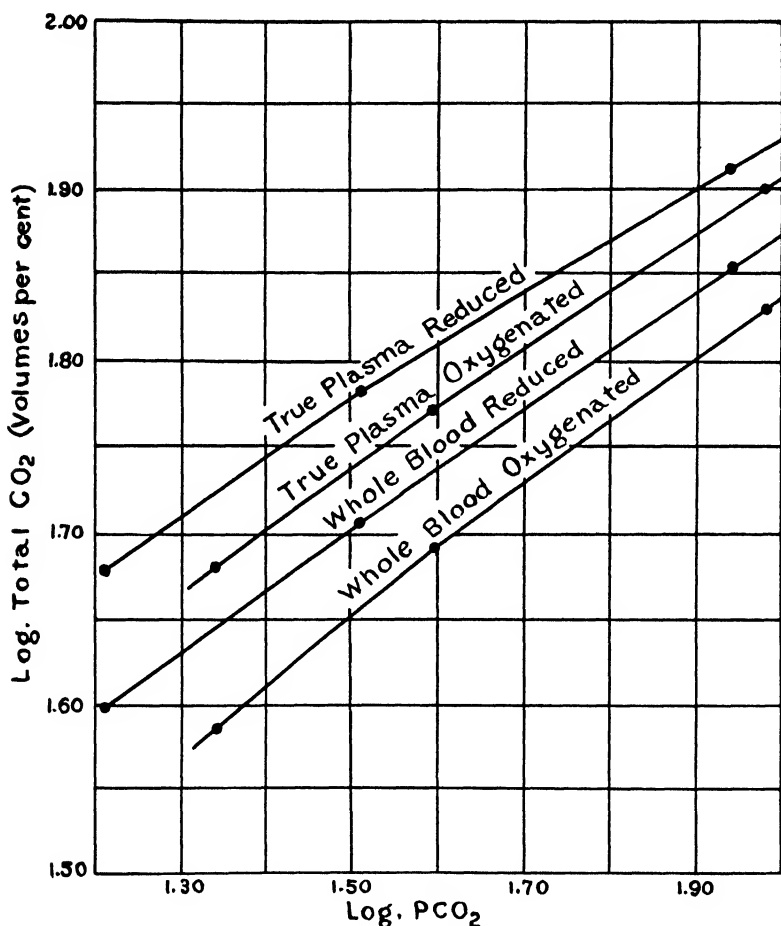


FIG 3. Logarithmic CO₂ dissociation curves.

distributed between strong acids (BCI) on the one hand and weak acids (BHCO₃ + BP) on the other and (2) in the proportion of cells and plasma. Probably slight physiological changes in the

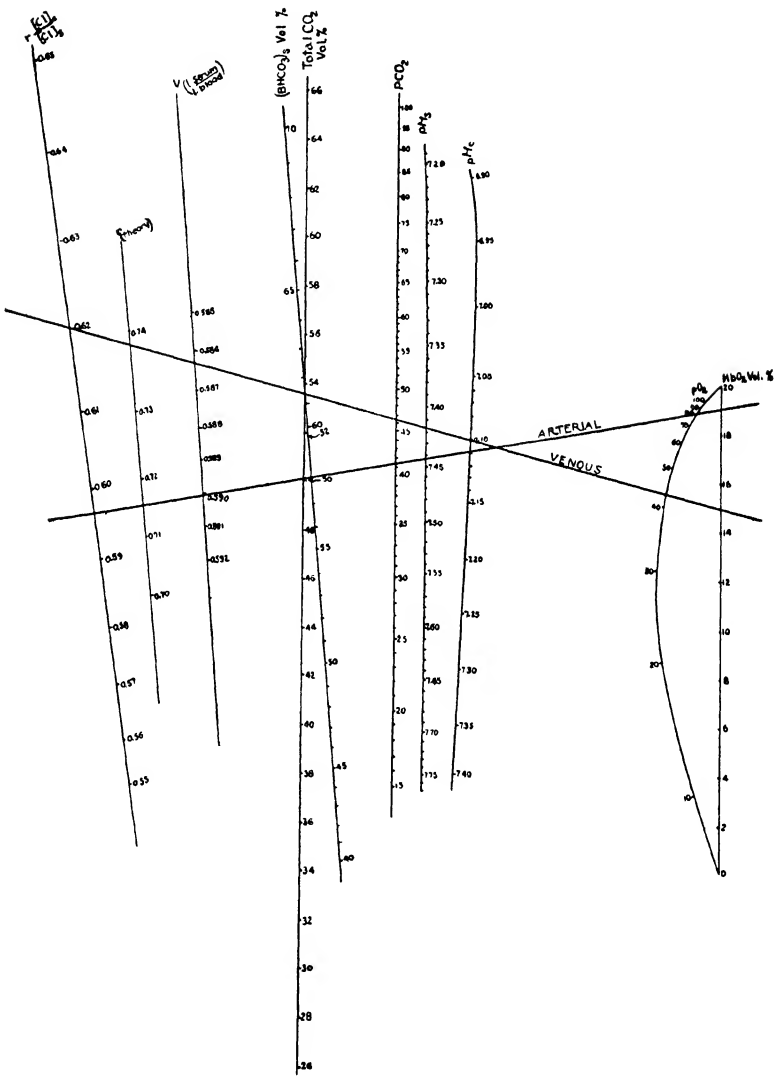


FIG. 4. Blood of C.V.C.

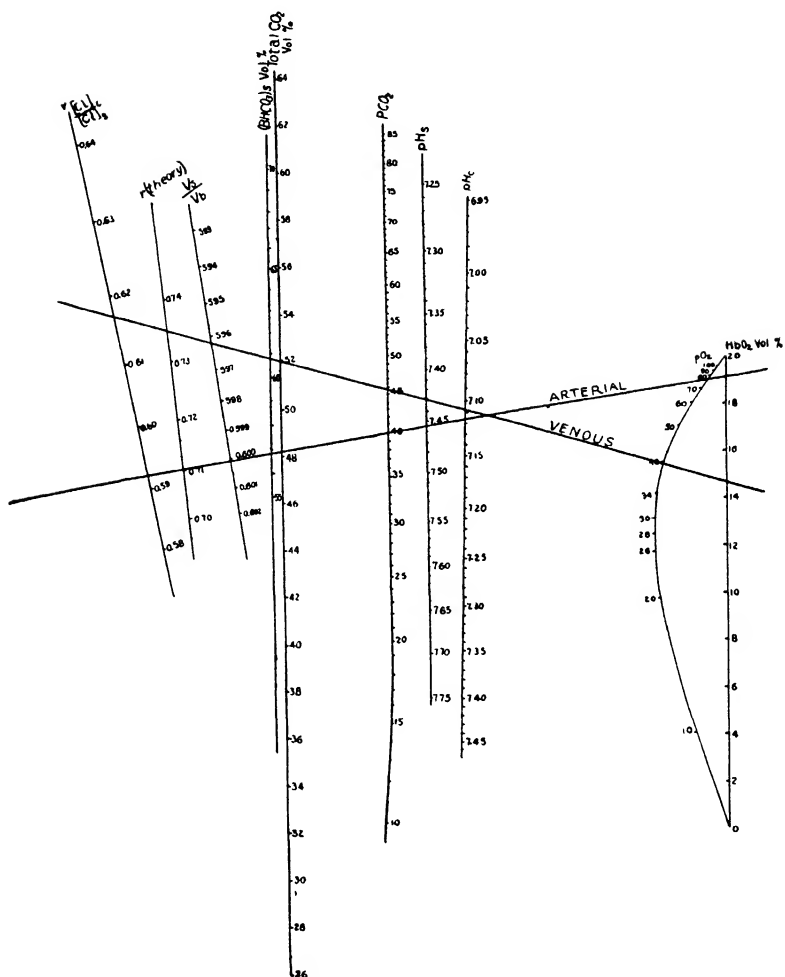


FIG. 5. Blood of A.V.B.

TABLE III.

Blood of C.V.C.

Concentration of hemoglobin . . 8.93 mm per liter of blood.

" " serum proteins 40.0 gm. " " "

R. Q. 0.82

	Arterial			Venous			Δ		
	Serum	Cells	Whole blood	Serum	Cells	Whole blood	Serum	Cells	Whole blood
H ₂ O cc. per l. blood . .	544	293	837	540	297	837	-4	+4	0
B m _M " " " . .	83.02	47.49	130.51	83.02	47.49	130.51	0	0	0
Cl " " " " . .	60.51	19.28	79.79	59.82	19.97	79.79	-0.69	+0.69	0
BP " " " " . .	7.22	22.18	29.40	7.13	20.87	28.00	-0.09	-1.31	-1.40
BHCO ₃ " " " " . .	15.29	6.03	21.32	16.07	6.65	22.72	+0.78	+0.62	+1.40
" " vol. per cent	34.25	13.52	47.77	36.00	14.93	50.93	+1.75	+1.41	+3.16
H ₂ CO ₃ m _M per l. blood	0.73	0.36	1.09	0.81	0.41	1.22	+0.08	+0.05	+0.13
" " vol per cent	1.63	0.80	2.43	1.81	0.91	2.72	+0.18	+0.11	+0.29
Total CO ₂ m _M per l. blood	16.02	6.39	22.41	16.88	7.06	23.94	+0.86	+0.68	+1.54
" " vol. per cent	35.88	14.32	50.20	37.81	15.84	53.65	+1.93	+1.52	+3.45
Free O ₂ m _M per l. blood			0.09			0.04			-0.04
" " vol. per cent . .			0.2			0.1			-0.1
Combined O ₂ m _M per l. blood		8.53	8.53		6.70	6.70		-1.83	-1.83
" " vol. per cent		19.1	19.1		15.00	15.00		-4.1	-4.1
Total O ₂ m _M per l. blood			8.62			6.74			-1.88
" " vol. per cent			19.3			15.10			-4.2
CO ₂ tension, mm. Hg . .			41.5			46.4			+4.9
O ₂ " " " " . .			79.0			43.0			-36.0
Volume, cc. per l. blood . .	590	410		586.3	413.7		-3.7	+3.7	0
pH	7.442	7.108		7.416	7.098		-0.026	-0.010	-0.024
r _{theory}			0.715			0.739			+0.024
r _{Cl}			0.596			0.620			+0.024

TABLE IV.
Blood of A.V.B.
 Concentration of hemoglobin 8.93 mm per liter of blood.
 " serum proteins 43.5 gm. " " "
 R. Q. 0.82

	Arterial			Venous			Δ		
	Serum	Cells	Whole blood	Serum	Cells	Whole blood	Serum	Cells	Whole blood
H ₂ O cc. per l. blood	550	282	832	546	286	832	-4	+4	0 0
B m _M " " "	82.71	45.96	128.67	82.71	45.96	128.67	0 0	0 0	0 0
Cl " " "	59.59	18.11	77.70	58.72	18.98	77.70	-0.87	+0.87	0 0
BP " " "	7.88	22.60	30.48	7.80	21.15	28.95	-0.08	-1.45	-1.53
BHCO ₃ " " "	15.23	5.25	20.48	16.19	5.83	22.02	+0.96	+0.58	+1.54
" vol. per cent	34.12	11.77	45.89	36.28	13.06	49.34	+2.16	+1.29	+3.45
H ₂ CO ₃ m _M per l. blood	0.71	0.34	1.05	0.80	0.39	1.19	+0.10	+0.05	+0.15
" vol. per cent	1.58	0.76	2.34	1.79	0.87	2.66	+0.21	+0.11	+0.32
Total CO ₂ m _M per l. blood	15.94	5.59	21.53	16.99	6.22	23.21	+1.06	+0.63	+1.69
" vol. per cent	35.70	12.53	48.23	38.07	13.93	52.00	+2.37	+1.40	+3.77
Free O ₂ m _M per l. blood			0.09			0.04			-0.04
" vol. per cent			0.2			0.1			-0.1
Combined O ₂ m _M per l. blood		8.57	8.57		6.56	6.56		-2.01	-2.01
" vol. per cent		19.2	19.2		14.7	14.7		-4.5	-4.5
Total O ₂ m _M per l. blood			8.66			6.60			-2.05
" vol. per cent			19.4			14.8			-4.6
CO ₂ tension, mm. Hg			40.0			45.4			+5.4
O ₂ " " "			78.0			40.0			-38.0
Volume, cc. per l. blood	600	400		596.2	403.8		-3.8	+3.8	0 0
pH.....	7.455	7.118		7.429	7.110		-0.026	-0.008	+0.025
r _{theory}			0.710						+0.024
r _{Cl}			0.592						

TABLE V.
C.V.C.

		Arterial.	Venous	Δ
Serum.				
H ₂ O	cc. per l. serum .	922 0	921 0	-1 0
B	m _M " " "	140 7	141 6	+0 9
Cl	" " " "	102 56	102 03	-0 53
BP	" " " "	12 24	12 16	-0 08
BHCO ₃	" " " "	25 92	27 41	+1 49
H ₂ CO ₃	" " " "	1 24	1 38	+0 14
Total CO ₂	" " " "	27 16	28 79	+1 63

Cells.

H ₂ O	cc. per l. cells	714 7	717 9	+3 2
B	m _M " " "	115 83	114 79	-1 04
Cl	" " " "	47 03	48 27	+1 24
BP	" " " "	54 10	50 45	-3 65
BHCO ₃	" " " "	14 71	16 08	+1 37
H ₂ CO ₃	" " " "	0 88	0 99	+0 11
Total CO ₂	" " " "	15 59	17 07	+1 48
Combined O ₂	" " " "	20 81	16 19	-4 38

A.V.B.

		Arterial.	Venous	Δ
Serum.				
H ₂ O	cc. per l. serum .	916 7	915 8	-0 9
B	m _M " " "	137 9	138 7	+0 8
Cl	" " " "	99 32	98 49	-0 83
BP	" " " "	13 13	13 08	-0 05
BHCO ₃	" " " "	25 39	27 16	+1 77
H ₂ CO ₃	" " " "	1 18	1 34	+0 16
Total CO ₂	" " " "	26 57	28 50	+1 93

Cells

H ₂ O	cc. per l. cells	705 0	708 3	+3 3
B	m _M " " "	114 90	113 8	-1 1
Cl	" " " "	45 27	47 00	+1 73
BP	" " " "	56 50	52 38	-4.12
BHCO ₃	" " " "	13 13	14 44	+1.31
H ₂ CO ₃	" " " "	0 85	0 96	+0.11
Total CO ₂	" " " "	13 98	15 40	+1.42
Combined O ₂	" " " "	21 43	16 25	-5.18

normal individual may produce changes in these variables which are as great as those which may be observed in comparing the blood of different healthy persons under similar conditions.

The horse blood presents a third difference in composition. There is in fact a significantly higher *concentration* of hemoglobin in the cells of the two men than in those of the horse. Similar differences, though smaller in magnitude, also occur in normal human blood, but considerable differences in this variable are perhaps either (1) specific or (2) pathological.

On the whole, the physicochemical mechanism of the blood which is involved in the transport of oxygen and carbon dioxide in normal individuals, even of different species, is constant to a very close approximation.

SUMMARY.

This paper consists of a comparative study of the respiratory functions of the blood in two normal resting men and in a horse.

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ON WALDEN INVERSION.

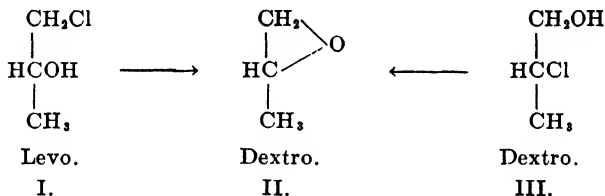
IX. ON THE MECHANISM OF HYDROLYSIS OF OPTICALLY ACTIVE PROPYLENE OXIDE.

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(Received for publication, March 16, 1927)

In order to understand fully the mechanism of the hydrolysis of propylene oxide, the configurational relationship of propylene oxide to propylene glycol must be established. Dextro-propylene oxide may be prepared from levo-1-chloro-2-hydroxypropane (I) or from dextro-1-hydroxy-2-chloropropane (III).



In these two transformations it is reasonable to assume that the first proceeds without inversion, inasmuch as reactions of substitution of the hydrogen on a hydroxyl group were shown by Kenyon and Phillips¹ and their coworkers to proceed normally, whereas substitution of a hydroxyl may bring about inversion.

In the present case the assumption may be substantiated by the following argument. From the work of Levene and Haller² and Levene and Waltj² it follows that levo-1-chloro-2-hydroxypropane has the configuration of levo-lactic acid; that is, it belongs

¹ Kenyon, J , Phillips, H., and Turley, H , *J. Chem Soc* , 1925, cxxvii, 399

² Levene, P A , and Haller, H. L , *J. Biol. Chem.*, 1925, lxxv, 49; 1926, lxxvii, 329. Levene, P A , and Waltj, A., *J. Biol. Chem* , 1926, lxxviii, 415.

to the *d* series and hence has the hydroxyl to the right. Hudson³ has demonstrated for a number of lactones of hydroxy acids that the direction of their rotation is determined by the allocation of the hydroxyl, those having the latter on the same side as levo-lactic acid (*d*) rotating to the right. On the basis of this rule, the configuration of levo-lactic acid (*d*) may be assigned to dextro-rotatory propylene glycol. Abderhalden and Eichwald⁴ held the opposite and erroneous view.

The question arises as to the reaction from (III) to (II). Abderhalden and Eichwald⁴ have shown that dextro-1-hydroxy-2-chloropropane is oxidized to levo-chloropropionic acid. This acid, on the basis of considerations of Clough,⁵ of Kenyon, Phillips, and Turley,¹ and of Levene and Mikeska,⁶ is configurationally related to levo-lactic acid (*d*); therefore the conclusion is justified that levo-1-chloro-2-hydroxypropane (I) and dextro-1-hydroxy-2-chloropropane (III) are configurationally related, and hence a second conclusion follows, that both reactions (one from (I) to (II) and the other from (III) to (II)) proceed without inversion. The significance of this observation will be referred to later. Thus, the reaction of formation of propylene oxide proceeds normally, the reverse reaction of hydrolysis either normally or abnormally depending upon the external conditions.

Abderhalden and Eichwald observed that the product obtained on hydrolysis of dextro-propylene oxide was at times dextrorotatory, at times levorotatory. As a rule, under the conditions employed by them, it was dextrorotatory. They justly remarked that the variations in direction of rotation could be explained either on the assumption of formation of some intermediate substances or on the assumption of Walden inversion in one case. These authors realized that the problem required further inquiry. Nevertheless, they assumed configurational relationship between levo-1-chloro-2-hydroxypropane and dextro-propylene glycol on the basis of the fact that the latter was formed from dextro-1-hydroxy-2-chloropropane and that it could also be transformed into levo-1-bromo-2-hydroxypropane. Their assumption was erroneous.

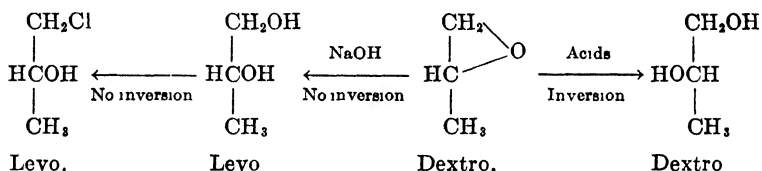
³ Hudson, C. S., *J. Am. Chem. Soc.*, 1910, xxxii, 338.

⁴ Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, 1918, li, 1312.

⁵ Clough, G. W., *J. Chem. Soc.*, 1918, cxiii, 526.

⁶ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1926, lxx, 365.

The progress of hydrolysis of dextro-propylene oxide has now been studied in some detail. It was found that the direction of rotation of the resulting glycol depends on the hydrogen ion concentration. A comparatively high hydrogen ion concentration of the hydrolysis medium leads from a dextrorotatory oxide to a dextrorotatory glycol. On the other hand, a high hydroxyl ion concentration leads to a levorotatory glycol. Inasmuch as from the previous work of Levene and Walti it is known that levo-1-chloro-2-hydroxypropane is configurationally related to levo-propylene glycol and hence that levorotatory propylene glycol is configurationally related to dextrorotatory propylene oxide, it now follows that hydrolysis with alkalis proceeds normally without inversion and that hydrolysis with acids proceeds with inversion.



In Table I are given the results of hydrolysis under the influence of various catalysts. It must be mentioned here that from the rotation of the reaction product alone, one is not justified in drawing conclusions regarding the direction of rotation of the glycol. The reaction product contains, in addition to the latter, condensation products of higher molecular weight, esters, etc. The conclusions formulated above were all based on experiments in which the glycol had been isolated.

Discussion of the Theories of Walden Inversion Occurring during Hydrolysis.

The type of Walden inversion described here has been observed in recent years on several occasions and every observer has emphasized the importance of it, inasmuch as it brings to our attention the possibility that every hydrolysis of an ester having one or both components optically active may lead to a change in the configuration of the active components. The same occurrence may take place on hydrolysis of lactones of optically active hydroxy acids.

Hence, it is important to be able to recognize those conditions of hydrolysis or of alcoholysis which lead to an inversion. The problem having been formulated only recently, there is at present comparatively little experimental material to permit comprehensive conclusions.

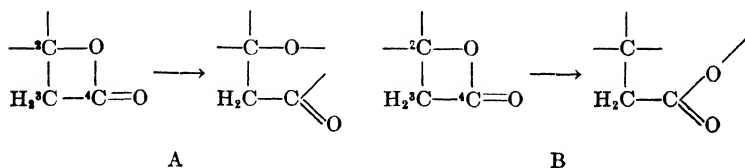
The two observations which are nearest in their character to the one described here are the one by Holmberg on propiolactone- β -carboxylic acid (malic acid β -lactone) and the other by Richard Kuhn and coworkers on ethylene oxide dicarboxylic acid.

Holmberg⁷ assumes that dextro-malic acid β -lactone is configurationally related to dextro-malic acid. Hydrolysis with alkalis leads to the dextro acid. Hence, according to Holmberg, the reaction proceeds without inversion. On the other hand, acid hydrolysis leads to a levo acid; hence, in the opinion of Holmberg, acid hydrolysis brings about an inversion.

If the assumptions of Holmberg are correct, then the conclusions of this author in regard to dextro-malic acid β -lactone coincide with ours in regard to propylene oxide and a general rule might be formulated connecting Walden inversion with cation hydrolysis and the normal reaction with anion hydrolysis. Such a hypothesis was in fact suggested by Gadamar⁸ as a general rule for Walden inversion.

In reality this comprehensive assumption is contradicted by recent observations of Kuhn and coworkers who found that in ethylene oxide dicarboxylic acid both the *cis* and the *trans* forms are hydrolyzed in the same sense by either acids or alkalis.

Kuhn and Ebel⁹ then offered the following explanation for the observations of Holmberg on dextro-malic acid β -lactone.



This explanation states that in reaction A (without inversion) the asymmetric carbon atom remains saturated all through the

⁷ Holmberg, B, *J prakt Chem*, 1913, lxxxviii, 456, 553

⁸ Gadamar, J, *J prakt Chem*, 1913, lxxxvii, 312

⁹ Kuhn, R, and Ebel, F, *Ber chem Ges.*, 1925, lviii, 919

In all discussions on Walden inversion it seems to us that too little attention was devoted to the possible interreaction of the groups of lesser polarity. The substitution or principal reaction takes place between the most polar groups of the reacting substances. For convenience of discussion these may be referred to as "groups of the first order" and the remaining atoms or radicles as "groups of the second order."

Assuming that the first step in any reaction of substitution is the formation of a complex in the sense of Kekulé, one may further assume that in this complex the tetrahedron of the asymmetric carbon atom possesses a different shape from that in the original substance and hence that the distances between the groups of the second order will be changed, thus enhancing or lowering the chance of their interreacting. The greater the original asymmetry of the tetrahedron, the greater will be the change produced in the complex.

With the background of these considerations, the outcome of a reaction of substitution may be viewed as the result of two reactions: one involving the two highly polar groups (groups of the first order) and the other involving the groups of lesser polarity (groups of the second order). When the velocity of the first reaction is much greater than that of the second, no Walden inversion will occur. When that of the second is greater, either inversion or racemization or both reactions may take place.

The advantage of this assumption lies in the fact that it permits an experimental investigation of the respective velocities: the first may be measured directly and the second may be studied by means of the velocities of racemization.

The outlined suggestion is in part the outcome of observations on Walden inversion in the series of secondary alcohols made in this laboratory by Levene and Mikeska which will be discussed later.

EXPERIMENTAL.

Optically Active Propylene Oxide.—Optically active propylene oxide was prepared according to our earlier publications. It may be mentioned that in one case the propylene oxide was prepared without a prior distillation of the crude propylene bromohydrin. Thus, 52 gm. of crude propylene bromohydrin (residue of the

TABLE I.

Experiment , No	Amount of acid or alkali	Amount of water	Amount of propylene oxide	Tempera- ture	Length of time.	Rotations in 1 dm tube.
		cc	cc	°C	hrs.	degrees
1	0.0800 gm <i>d</i> -tartaric acid	7.5	4.5 ($\alpha = +9.55^\circ$)	64-65	16	+2.30
2	0.10 cc formic acid	2.0	1.2 "	64-65	16	+1.57
3	2.5 " N H ₂ SO ₄		1.0 "	46	17	+1.16
4	2.5 " " H ₃ PO ₄		1.0 "	46	17	+1.28
5	0.034 gm quinic acid	2.5	1.0 "	46	17	+1.00
6	0.030 " camphor-sulfonic acid	2.5	1.0 "	64-65	17	+1.54
7	0.042 " oxalic acid	2.5	1.0 "	64-65	17	+1.34
8	0.1659 " "	5	2.0 ($\alpha = +8.51^\circ$)	33-34	16	+1.13
9	0.3318 " "	5	2.0 "	33-34	16	+1.25
10	0.6626 " "	5	2.0 "	33-34	16	+1.25
11	0.220 " "	5	5.0 "	33-34	16	+1.84
12	10 cc N HCl		3.2 "	50	18	-0.32*
13	2.5 " 2 N HCl		1.0 ($\alpha = +10.50^\circ$)	65-80	17	-0.01*
14	2.6 " 4 "		1.0 "	65-80	17	-0.80*
15	2.5 " N NaOH.		1.0 "	64-65	17	-7.50
16	4 " " "		2.0 "	35-36	15	-8.85
17	5 " " "		2.5 "	36-37	18	-8.02

* The small levorotation in these experiments was due to the presence of small quantities of levo-chlorohydrin, inasmuch as the glycol isolated was dextrorotatory.

chloroform extract obtained on treatment of 40 gm. of propylene glycol ($[\alpha]_D = -13.71^\circ$) with 23 gm. of potassium hydroxide and 30 cc. of water gave 18 gm. of propylene oxide ($[\alpha]_D = +8.51^\circ$).

Hydrolysis of Optically Active Propylene Oxide.—All experiments on the hydrolysis of propylene oxide were carried out in sealed tubes. The conditions employed in each experiment are recorded in Table I. After cooling to room temperature the rotations of the aqueous solutions were taken in a 1 dm. tube.

Dextro-Propylene Glycol from Dextro-Propylene Oxide by Means of Tartaric Acid.—From Experiment 1 the dextro-propylene glycol was isolated by evaporating the aqueous solution under reduced pressure. The residue was taken up in alcohol and the solution was concentrated under reduced pressure until all water and alcohol were removed; the glycol was then distilled at 92°C . and 15 mm. It showed a rotation of $\alpha = +4.11^\circ$ without solvent. In order to confirm this result 1 gm. of the propylene glycol obtained in this manner was transformed into the di-(phenylurethane) by adding 3.3 gm. of phenylisocyanate and heating at $100\text{--}115^\circ\text{C}$. for 1 hour. The urethane was isolated as previously described. It had a melting point of $145\text{--}146^\circ\text{C}$. and analyzed as follows:

0.1000 gm substance required 6.02 cc 0.1 N HCl	
$\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_4$	Calculated N 8.91
	Found " 9.03.

The rotation of the substance in absolute alcohol was as follows:

$$[\alpha]_D^{21} = \frac{-0.19^\circ \times 100}{1 \times 4} = -4.75^\circ$$

Dextro-Propylene Glycol from Dextro-Propylene Oxide by Means of Oxalic Acid.—From the reaction products of Experiments 8, 10, and 11, dextro-propylene glycol was isolated in the manner described above.

In Experiment 8 the isolated propylene glycol had the following rotation in water.

$$[\alpha]_D^{20} = \frac{+0.52^\circ \times 100}{1 \times 17.2} = +3.02^\circ$$

In Experiment 10 the propylene glycol had the following rotation in water.

$$[\alpha]_D^{20} = \frac{+0.51^\circ \times 100}{1 \times 16.4} = +3.10^\circ.$$

In each of these two experiments the yield of propylene glycol was 1.3 gm.

In Experiment 11 the rotation of the propylene glycol in water was as follows:

$$[\alpha]_D^{20} = \frac{+0.61^\circ \times 100}{1 \times 16.9} = +3.60^\circ.$$

The rotation of the propylene glycol without solvent was $\alpha_D = +3.25^\circ$. In the latter series of experiments it was observed that a neutral water-insoluble, ether-soluble compound was formed in small quantities. This compound probably represents a neutral ester of oxalic acid and propylene glycol.

Dextro-Propylene Glycol from Dextro-Propylene Oxide by Means of Hydrochloric Acid.—In Experiments 12, 13, and 14 with hydrochloric acid, the reaction mixture was levorotatory. As this reaction mixture was neutral toward litmus and did not contain free chlorine ions, the hydrochloric acid was apparently bound to the oxide. Thus, dextro-propylene oxide forms levo-propylene chlorohydrin. In order to isolate the propylene glycol the calculated amount of 2 N hydrochloric acid was slowly added in the cold to 3 cc. of dextro-propylene oxide ($\alpha = +9.5^\circ$). The rotation of the solution was $\alpha = -0.71^\circ$ in a 1 dm. tube. The solution was concentrated under diminished pressure and the viscous residue made up to 2.5 cc. with water. The rotation was $\alpha = +2.03^\circ$. The solution was again concentrated until all water was removed and from the residue the urethane of propylene glycol was prepared. It melted at $145\text{--}146^\circ\text{C.}$ and analyzed as follows:

0.0500 gm substance required	3.17 cc. 0.1 N HCl
$\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_4$	Calculated N 8.91.
	Found " 8.88

Alkaline Hydrolysis of Optically Active Propylene Oxide.—The optically active propylene oxide in Experiment 16 was hydrolyzed with normal sodium hydroxide solution. After the polarimetric reading had been taken the solution was made neutral to litmus

with hydrochloric acid and made up to 10 cc. with water. The rotation was $\alpha = -5.15^\circ$. The solution was evaporated under diminished pressure; the residue was taken up in absolute alcohol, freed from the latter, and the propylene glycol was then distilled at 92° and 15 mm.; the yield was about 0.8 gm. The rotation was as follows:

$$[\alpha]_D^{20} = \frac{-1.55^\circ \times 100}{1 \times 6.20} = -25.0^\circ.$$

After the distillation of the propylene glycol, a residue was left which gave the following rotation in water.

$$[\alpha]_D^{20} = \frac{-3.53^\circ \times 100}{1 \times 9.34} = -37.8^\circ.$$

A certain amount of this product in the propylene glycol just described caused its higher rotation.

In order to find the nature of this residue, Experiment 17 was started and worked up as stated above. The propylene glycol obtained from this experiment had a rotation in water of

$$[\alpha]_D^{20} = \frac{-1.52^\circ \times 100}{1 \times 6.74} = -22.55^\circ.$$

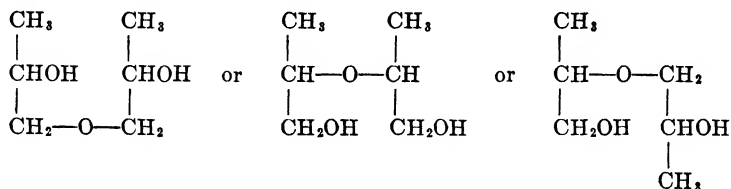
To 0.25 gm. of this propylene glycol was added 0.8 gm. of phenylisocyanate and the mixture was heated for 1 hour at $100-105^\circ$. After cooling, the solid product was washed in a mortar with very little ether, filtered under suction, and crystallized from dilute alcohol. The melting point was $145-146^\circ\text{C.}$ and the substance analyzed as follows

0.0110 gm substance	0.962 mg N
	$\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}_4$ Calculated. N 8.91.
	Found " 8.75.

The rotation of the di-(phenylurethane) in absolute alcohol was

$$[\alpha]_D^{20} = \frac{+0.43^\circ \times 100}{1 \times 3.60} = +11.94^\circ.$$

The substance which distilled (after the propylene glycol) at 105–110°C. and 15 mm. probably represents



0.0936 gm. substance: 0.1808 gm. CO_2 and 0.0886 gm. H_2O .

$\text{C}_6\text{H}_{14}\text{O}_3$. Calculated. C 53.73, H 10.45.
Found " 52.67, " 10.69.

The rotation of the substance in water was

$$[\alpha]_D^{20} = \frac{-4.38^\circ \times 100}{1 \times 11.34} = -38.62^\circ$$

Preparation of Dextro-Propylene Glycol of Higher Optical Activity.—5 cc. of dextro-propylene oxide ($\alpha = +8.51$) were added to 8 cc. of anhydrous formic acid and the glycol was isolated according to the directions of Abderhalden and Eichwald. 3 gm. of dextro-propylene glycol were obtained which had a rotation in water of

$$[\alpha]_D^{20} = \frac{+1.78^\circ \times 100}{1 \times 15.9} = +11.2^\circ$$

The rotation of the glycol without solvent was about $+8^\circ$.¹¹

This dextro-propylene glycol was transformed into dextro-propylene bromohydrin by the methods which have been described previously. The rotations of the dextro- and of the levo-propylene bromohydrin were taken in benzene and chloroform.

The rotations of the dextro-propylene bromohydrin were as follows:

$$1. \text{ In chloroform solution } [\alpha]_D^{20} = \frac{+0.77^\circ \times 100}{1 \times 14.2} = +5.42^\circ.$$

$$2. \text{ In benzene solution } [\alpha]_D^{20} = \frac{+0.59^\circ \times 100}{1 \times 14.8} = +3.99^\circ.$$

¹¹ On account of layer formation the rotation could not be taken very accurately.

The rotations of the levo-propylene bromohydrin were:

1. In chloroform solution $[\alpha]_D^{20} = \frac{-1.62^\circ \times 100}{1 \times 20.0} = -8.10^\circ$.
2. In benzene solution $[\alpha]_D^{20} = \frac{-1.22^\circ \times 100}{1 \times 20.9} = -5.83^\circ$.
3. Without solvent $[\alpha]_D^{20} = \frac{-2.32^\circ}{1 \times 1.599} = -1.45^\circ$

Levo-propylene bromohydrin was prepared by the addition of hydrobromic acid to dextro-propylene oxide. To 14 gm. of propylene oxide ($\alpha = +10.44^\circ$) was added slowly under cooling a solution of 46 gm. of hydrobromic acid (sp. gr. 1.45) in 60 cc. of water. The bromohydrin was taken up in chloroform, neutralized with sodium carbonate, dried with sodium sulfate, and distilled under diminished pressure. 10 gm. of levo-propylene bromohydrin were obtained.

The substance analyzed as follows

0.1221 gm substance	0.1642 gm AgBr		
	C ₃ H ₇ OBr	Calculated	Br 57.55
		Found	" 57.23

IS INSULIN INACTIVATED BY GLUCOSE?

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(Received for publication, February 26, 1927.)

A year and a half ago, Professor Murlin reported in a preliminary communication from this laboratory that insulin was inactivated by incubation at 37° for 1 or 2 hours with 0.3 to 1.0 per cent glucose (1). This conclusion was based upon observations made by himself and Allen during the summer months of 1925. Allen having left this laboratory the latter part of August, Professor Murlin requested the writer to amplify the experimental data for the purpose of developing a related problem. Although the results which Murlin and Allen had obtained appeared perfectly consistent, there was a possibility that the summer heat had interfered with the accuracy of the animal tests. Accordingly the experiments about to be reported were begun in the fall of that year and were continued with some interruption from October to January, 1926.

The insulin used in the majority of the experiments reported here was prepared according to the method of Allen and Murlin (2). The improvements in the method instituted in this laboratory which had been used in the preparation of the material described by Murlin as A₃P₅ were followed. In some of the later experiments in the present work a more highly purified insulin was used which had been prepared according to the method of Abel (3) from a concentrated solution of Lilly'sletin. This purified product contained approximately 30 clinical units per mg.¹

¹ The method of Abel in purifying insulin has proved to be quite effective in our hands although some batches of letin seem to behave somewhat differently from the description in his publication. In one case, the fraction insoluble in phenol was practically nil. The potencies claimed by Abel are quite easily obtainable by his method, and using his brucine modification (4), we have recently obtained a preparation 0.01 mg. per kilo of which caused lowering of the blood sugar in rabbits of 40 mg.

The insulin and Merck's c p glucose in quantity to give the desired concentration were dissolved in the required amount of dilute hydrochloric acid or other medium used. The mixture was brought to a temperature of 37-40° and incubated at that temperature for the designated time. It was then injected subcutaneously into rabbits as soon as possible after incubation. Blood samples were taken from the marginal ear vein before the injection and 2

TABLE I.

Fall in Blood Sugar from Insulin Incubated with Glucose in HCl pH 2

Preparation No	Rabbit No	Incubation time	Concentration of glucose	Blood sugar (mg per 100 cc.).		
				Before	After	Decrease
		<i>hrs</i>	<i>per cent</i>			
3a	4	2	0.5	108	68	40
	4	2	Control	105	51	54
	1	24	0.5	113	55	58
	2	24	0.5	115	67	48
	5	24	Control	125	90	35
	6	24	"	108	88	20
	3	2	1.0	95	57	38
	9	2	1.0	125	65	60
	8	2	Control	115	59	56
	10	2	"	100	64	36
	1	48	1.0	100	56	44 Average test 48
	2	48	Control	124	60	64 " control 44
3b	10	1	0.5	110	93	17
	9		Control.	108	78	30
	3		0.5	112	76	36
	8		0.5	105	75	30
	5		Control	115	95	20 Average test 28
	6		"	130	90	40 " control 30

hours later. The samples were run simultaneously with the controls for the sugar concentration, according to the method of Folin and Wu (5). For control, insulin and glucose solutions of the same concentrations used in the test experiments were incubated separately. The insulin solution was then injected subcutaneously on one side of the animal and the glucose solution on the other.

The first preparation used in the attempt to repeat Murlin and

Allen's observations was Preparation 3a (purified by two precipitations with amyl alcohol). It was incubated with 0.5 and 1.0 per cent glucose solutions at 37° for 2, 24, and 48 hours. Another preparation, No. 3b, was also tried, incubating it at 37° for 1 hour with 0.5 per cent glucose. 0.5 cc. of a hydrochloric acid solution of pH 2 containing 2 mg. per cc. in the case of Preparation 3a and 5 mg. per cc. in the case of Preparation 3b was injected per kilo. In this series six experiments were run using twenty rabbits. Although the procedure of Murlin and Allen was closely duplicated, no inactivation could be demonstrated within experimental errors. The results are given in Table I. As has been noted many times before, the response of different rabbits with the same insulin preparation varies greatly. Certain rabbits of the same stock and kept under the same conditions seem to be exceptionally sensitive to insulin. On the other hand, some are quite resistant. Controls were run occasionally in which nothing was injected, in order to check up on the determination of the blood sugar and the normal variation during the 2 hour interval. The original and the 2 hour sample consistently agreed within 10 mg. In all the experimental work the rabbits were fasted 18 hours before the test.

However, Murlin and Allen had found that their unpurified salt precipitate was not inactivated nor was the sample of unpurified Lilly'sletin. It was thought, therefore, that a more purified preparation might be inactivated. The purification with amyl alcohol precipitation accordingly was carried further. It was reprecipitated with amyl alcohol 3 more times and then extracted with 95 per cent ethyl alcohol. This product, Preparation 3c, corresponds to the A_3P_6 used by Murlin and Allen and compares favorably with it in potency. 1 mg. per kilo of this material gave a drop of 55, 45, and 50 mg. of glucose per 100 cc. of blood in three rabbits respectively.

20 mg. of this material were dissolved in 10 cc. of hydrochloric acid of pH 2. To this 100 mg. of glucose were added making a 1.0 per cent solution. The mixture was then incubated for 2 hours. As usual controls were run in the manner described. The results are tabulated in Table II. In Experiment 9 the controls showed a greater fall in blood sugar than the test animals but a repetition of this experiment (No. 10) failed to show a significant difference between control and test. An experiment (No. 11) was then run

using a 0.5 per cent glucose concentration, other conditions being the same as in the foregoing experiment. The rabbits used in this series and numbered above twelve were rabbits that had been in the laboratory only a couple of weeks. They had not been used before and seemed to give a greater response to insulin than those that had been in the laboratory and had been used repeatedly

TABLE II.

Decrease in Blood Sugar of Rabbits Injected with Preparation 3c Dissolved in HCl pH 2 and Incubated with Glucose for 2 Hours at 39°.

Experiment No	Rabbit No.	Concentration of glucose	Blood sugar (mg per 100 cc)		
			Before	2 hrs after	Decrease
		<i>per cent</i>			
9	2	1 0	108	75	33
	4	1 0	103	81	22
	1	Control.	115	60	55 Average test 28
	7	"	115	70	45 " control 50
10	1	"	102	60	42
	4	"	103	83	20
	9	"	111	73	38
	2	1 0	106	83	23
	7	1 0	94	57	37
	10	1 0	103	75	28 Average test 29
	8	1 0	98	69	29 " control 33
11a	5	Control.	98	70	28
	3	0 5	88	59	29 Average test 20
	6	0 5	100	89	11 Control 28
11b	13	Control.	110	55	55
	15	"	95	50	45
	14	0 5	110	70	40
	16	0 5	92	46	46 Average test 41
	17	0 5	115	78	37 " control 50

although not at too frequent intervals. It is possible that with repeated administrations of insulin the animals develop a greater resistance to the insulin.

Experiments were then arranged to vary the hydrogen ion concentration. In one set of experiments the material was dissolved in hydrochloric acid of pH 4 instead of pH 2. In another series

0.0001 N NaOH was used giving a pH of about 9.5. In some of these tests the individual variations in the response of the rabbits were quite erratic. The results, however, do not lend support towards a demonstration of inactivation of insulin. In Table III it will be noticed that the controls show a drop greater than the test animals. However, Rabbits 20 and 23 proved to be very sensitive to insulin in later work. The average of the test animals is about the same as that obtained in the other controls with which

TABLE III.

Decrease in Blood Sugar of Rabbits Injected with Preparation 3c Incubated with Glucose for 2 Hours at 39°

Experiment No.	Rabbit No	Concentration of glucose	Solution	Blood sugar (mg per 100 cc)		
				Initial	2 hrs	Decrease
		<i>per cent</i>				
12	20	Control	HCl pH 4	119	45	74
	18	"		120	103	17
	23	"		123	47	76
	19	0.5		113	78	35
	21	0.5		108	92	16
	22	0.5		107	72	35
	24	0.5		97	56	41
	25	0.5		107	72	35
						Average test 32 " control 56
13	13	Control	NaOH pH 9.4	112	59	53
	15	"		92	57	35
	1	"		94	51	43
	16	0.5		93	53	40
	2	0.5		93	48	45
						Average test 43 " control 44

this preparation was used, so we do not feel that this difference is significant. In the slightly alkaline medium there is no sign of inactivation.

At this time another preparation, No. 4, was used. This had been prepared similarly to Preparation 3c but had been reprecipitated with amyl alcohol seven times. This preparation was incubated with enough glucose to make a 0.5 per cent solution; in one case the material was dissolved in HCl of pH 2, in another in HCl of pH 4, and in a third, in a M/15 phosphate buffer solution of pH 7.4. The data presented in Table IV indicate again no inactivation.

Insulin Inactivated by Glucose?

TABLE IV.

Decrease in Blood Sugar of Rabbits Injected with Preparation 4 Incubated with Glucose for 2 Hours at 39°.

Experiment No	Rabbit No	Concentration of glucose.	Solution	Blood sugar (mg per 100 cc).		
				Initial	2 hrs	Decrease
14	1	Control	m/15 phosphate buffer pH 7.4	91	55	36
	17	0.5		110	75	35 Average test 32
	19	0.5		109	80	29 Control 36
15	15	Control.	HCl pH 4	105	55	50
	23	"		89	30	59
	25	"		106	57	49
	16	0.5		92	61	31 Average test 52
	20	0.5		109	39	70 " control 52
	24	0.5		96	40	56

TABLE V.

Decrease in Blood Sugar of Rabbits Injected with the Purified Insulin Dissolved in HCl pH 2 and Incubated for 1 Hour with Enough Glucose to Make a 0.5 Per Cent Solution.

Experiment No	Rabbit No	Glucose 0.5 per cent	Blood sugar (mg per 100 cc)		
			Initial	2 hrs	Decrease
17	17	Control.	112	46	66
	26	"	102	47	55 Average 60
	19	Merck.	105	70	35
	27	"	112	65	47
	28	"	103	53	50 Average 44
	13	"Brown."	107	30	77
	29	"	96	35	61 Average 69
18	30	Control.	120	70	50
	32	"	112	44	68
	34	"	122	67	55
	31	Merck.	118	36	82
	33	"	134	94	40
	35	"	115	80	35 Average test 54
	37	"	105	45	60 " control 57

The insulin used in the remainder of the experiments was the purified iletin mentioned above. 0.05 mg. per kilo of this preparation caused an average drop in rabbits of about 60 mg. of glucose per 100 cc. of blood. This preparation is about 20 times as potent as the A_3P_5 . In the first experiment with this product another brand of glucose was used; namely, a sample of fairly pure glucose of a brownish color and granular in nature. This was done to see if it

TABLE VI.

Decrease in Blood Sugar of Rabbits Injected with the Purified Iletin Incubated for 2 Hours with Glucose and $FeCl_3$ in Phosphate Buffer pH 6.8.

Experiment No	Rabbit No	Concentration of glucose	Blood sugar (mg per 100 cc)		
			Initial	2 hrs	Decrease
19	32	<i>per cent</i> Control.	98	50	48
	34	"	115	58	57
	33	0 5	88	57	31
	35	0 5	110	75	35 Average test 42
	36	0 5	114	53	61 " control 52
20, 21	17	0 5	115	95	20
	19	0 5	100	63	37
	20	0 5	104	44	60
	23	0 5	111	40	71 Average test 52
	25	0 5	110	40	70
22	27	Control.	117	62	55
	29	"	106	30	76
	35	"	113	75	38
	26	0 5	108	52	56
	28	0 5	103	52	51
	31	0 5	107	60	47 Average test 51
	32	0 5	101	51	50 " control 56

were possible that there might be something present in an impure glucose that would inactivate insulin. The results, however, show no signs of inactivation with the brown glucose and although the decreases obtained from the insulin incubated with the c.p. glucose are greater than the control, the difference is not enough to lend much support to inactivation (Experiment 17, Table V). Nevertheless, it was thought worth while to run another series of

rabbits with the c.p. glucose, using the same conditions as above. The results of this experiment (No. 18, Table V) show no sign of inactivation.

Thinking that it might be possible that iron salts are necessary for the inactivation of insulin by glucose, experiments were run adding a little ferric chloride to the incubation mixture of insulin and glucose. 2 cc. of a solution containing 0.4 mg. of insulin of HCl pH 2 were added to 2 cc. of 0.01 N NaOH and 4 cc. of M/15 phosphate buffer solution of pH 6.8. A few drops of 0.01 per cent ferric chloride were added. 5 cc. of this solution were added to 25 mg. of glucose. The mixture was then brought to a temperature of 39° and incubated at that temperature for 2 hours. 0.5 cc. of the solution was injected per kilo. As usual controls were run using 0.5 per cent glucose and insulin, incubated separately. The results (Experiment 19, Table VI) from this experiment might be interpreted as indicating a slight reduction in the potency of the insulin by the glucose. However, when more rabbits were used the difference between controls and test animals grew very much smaller. In Experiments 20 to 22, Table VI, are given the results from other tests, duplicating the experimental conditions of the foregoing experiment. Here the average drop in blood sugar is practically the same in both test and control

SUMMARY.

Altogether twenty-two separate experiments were run in the attempt to demonstrate an inactivation of insulin by glucose, using on an average five rabbits for each experiment. The decrease in blood sugar obtained in the test animal was often slightly less than that in the control. The difference however was not great enough to be interpreted as indicating definitely an inactivation, in view of the great variability in the response of rabbits to insulin. In certain experiments where it appeared as if inactivation had resulted, the use of more rabbits negatived the difference between control and test. The average decrease in blood sugar of 60 tests was 41.8 mg. per 100 cc. of blood; the average of 44 controls was 45.1.

The writer wishes to express his appreciation to Professor J. R. Murlin for his interest and helpful suggestions during the course of this investigation.

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THE EFFECTS OF PROTEIN SPLIT-PRODUCTS UPON METABOLISM.

I. THE FRACTION EXTRACTED BY AND PRECIPITATED IN BUTYL ALCOHOL (FRACTION I).

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INTRODUCTION.

Following an observation made by one of us (1) that the specific dynamic action of six different proteins was practically identical, in spite of their varying amino acid content, an attempt was made to explain this phenomenon, which was an apparent contradiction of Lusk's theory (2) that the specific dynamic action of proteins was due chiefly to glycine and alanine. In the course of their experiments directed towards this end, Weiss and Rapport (3) made the observation that when glycine or alanine was given with either gelatin or casein, the specific dynamic action of the amino acid was nullified, and the resulting metabolism was as though the protein had been given alone. Yet when the amount administered of an individual protein was increased, the metabolism increased proportionately, and when two proteins were fed at the same time, or when glycine and alanine were given together, the increased cellular oxidation resulting from the ingestion of the two substances was summated. It was concluded that the amino acids contained in a protein did not, when that protein was fed to a dog, undergo the same metabolism as did the same substances when given in the form of the free amino acids. Moreover, this apparent difference was not due to reactions taking place in the gastrointestinal tract, because the lack of summation observed when glycine and gelatin were fed simultaneously was equally in evidence when glycine was injected either subcutaneously or intravenously immediately after the gelatin had been eaten by the animal.

It appeared that a study of the stimulating properties of the split-products of protein digestion might throw light upon some of the questions raised by the above mentioned work. In a recent paper, one of us (4) has shown that the *in vitro* hydrolysates of gelatin behave like their parent protein. When administered in quantities containing the same amount of nitrogen, the sulfuric acid hydrolysate of gelatin had practically the same stimulating effect as did the gelatin itself, while the trypsin hydrolysate produced only a slightly greater effect. Moreover, like gelatin, the hydrolysates summated with the protein itself, and inhibited the specific dynamic action of glycine, whether the latter was given by mouth or intravenously.

The method of Dakin (5) for the separation of proteins into four fairly distinct fractions by butyl alcohol extraction, seemed to offer a readily feasible method for a more detailed study of the protein split-products. The present paper will concern itself with certain aspects of the investigation of the first of these fractions, namely, the one extracted from the sulfuric acid hydrolysate by butyl alcohol and precipitated in it. This fraction consists of the monoamino, monocarboxylic acids, and may contain glycine, alanine, serine, cystine, hydroxyproline, valine, leucine, phenyl-alanine, and tyrosine. Tryptophane, which might otherwise be present, is destroyed by the acid hydrolysis. The fraction was of especial interest to us on account of its content of glycine and alanine.

Technique.

A. Measurement of the Metabolism.

The method was the same as that described in an earlier paper (4). The apparatus was a modification of the closed system described by Benedict and Homans (6). The CO₂ and water produced by the animal were absorbed by soda-lime and sulfuric acid respectively, and measured directly. The O₂ was measured by weighing the amount which had to be admitted from a small tank during the course of an experimental period (usually 1 hour) in order to keep the volume of the system constant, and making the necessary corrections for changes in the barometric pressure, the temperature of the air in the box containing the animal, and

the amount of CO_2 and water vapor present in the system. The two latter were determined by analyzing samples of the circulating air at the beginning and end of the period.

The same animal, a well trained dog, which had been the subject of many previous experiments, was used throughout. It usually lay very quietly in the box for a period of as long as 5 hours, and very few periods had to be discarded on account of the animal's restlessness. On a diet of 75 gm. of cracker meal, 75 gm. of beef heart, 15 gm. of lard, and 5 gm. of bone ash, the dog maintained a constant weight of between 6.5 and 6.7 kilos.

With rare exceptions the dog ate both the proteins and the protein fractions without reluctance, the latter being made into a thick paste with water. Glycine had to be given in solution by stomach tube.

Calculation of the experiments was made using the ordinary protein factors, as it has been shown (1) that the error due to the fact that other proteins were ingested is inappreciable. In the case of the experiments where the fraction extracted by butyl alcohol was administered an estimate of the percentage of each amino acid metabolized would in any case have been a mere guess. In the experiments where glycine alone was given, the factors calculated by Lusk (7) were used.

B Preparation of the Amino Acid Fraction Extracted by and Precipitated in Butyl Alcohol.¹

We followed the method of Dakin in the following manner. Baker's gelatin or Merck's commercial casein, in batches of 700 gm., was boiled for 50 to 60 hours with 875 cc. of sulfuric acid and 2600 cc. of water. The solution was then diluted with water and neutralized with calcium carbonate, being alkaline to Congo red and faintly acid to litmus. Calcium was used instead of barium, in spite of the greater solubility of calcium sulfate, because of the danger that traces of the barium might remain in combination with the amino acids. Previous experience with hydrolysates neutralized with barium had demonstrated an apparent toxicity, showing itself in vomiting when the neutralized product had been

¹ We wish to express our thanks to Mr. J. T. Wolf for assistance rendered in the preparation of the butyl alcohol extracts.

given to dogs. This evidence of toxicity was obviated by the use of calcium.

The calcium sulfate was filtered off after the neutralization was complete and the filtrate then evaporated to a volume of about 4 liters and extracted with butyl alcohol. The latter was distilled over from a 1 liter, round bottom, Pyrex flask into a tall cylinder containing about 500 cc of the solution to be extracted, covered by a column of butyl alcohol about 5 inches in height, into which the return tube to the butyl alcohol flask dipped. Several such extraction batteries were set up, the cylinders holding the hydrolysates being surrounded by a water bath at a temperature of between 60–80°C.

At the end of each day the granular precipitate in the butyl alcohol flask was filtered off, washed with butyl alcohol and ether to free it from pigment, and dried at 45°C. in an oven. The precipitate contains most of the monoamino, monocarboxylic acids and is the fraction with which this report will chiefly concern itself.

Each cylinder of solution was extracted, in the case of casein, for from 30 to 40 hours. By this time the amount of precipitate still accumulating in the butyl alcohol flask was negligible. We found, as did Dakin, that in the case of gelatin precipitate continued to accumulate even after the extraction had been carried on for a long period of time. No attempt, therefore, was made to carry this to completion, the extraction being stopped in each cylinder at the end of about 10 days. According to Dakin, the slowly extracted acids are glycine and hydroxyproline, which appear in other fractions.

EXPERIMENTAL.

A. Basal Metabolism.

Six determinations of the basal metabolism were made during the course of this work, and betrayed the same remarkable constancy that has been noted by Lusk and DuBois (8). Over a period of 3 months, the basal heat production per hour varied around a mean of 12.4 calories with a maximum deviation of 0.4 calories, or 3.8 per cent. The mean variation was +2.2 per cent and -1.6 per cent, almost the margin of error of the instrument (Table I).

In experiments performed on this dog in the spring of 1926, the basal metabolism was found to be 13.67 calories. This represents a drop in the heat production during the intervening interval—from April, 1926, to November, 1926—of about 10 per cent. It was not due to the phenomenon of cage life, to which attention has been called by Lusk (7), but probably to the fact that the animal, while apparently fully grown in the earlier experiments, had not arrived at full maturity. Nevertheless, the reactions to foodstuffs in the earlier research, and in the one reported

TABLE I
Basal Metabolism

Date	Experiment No	Urinary N	R Q	O ₂ per hr	Calories per hr
<i>1926</i>		<i>gm</i>		<i>gm</i>	
Oct. 27	32	0 098	0 84	3 74	12 51
Nov 8	39	0 102	0 80	3 67	12 15
" 18	44	0 107	0 90	3 72	12 67
Dec. 1	50	0 100	0 86	3 67	12 30
" 27	61	0 099	0 84	3 66	12 20
<i>1927</i>					
Jan. 24	69	0 106	0 89	3 72	12 57
Average		0 102	0 85	3 70	12 40

Mean deviation = +2.2 and -1.6 per cent

Maximum deviation = 3.8 per cent.

in this paper, were not markedly different. For example, an amount of gelatin which resulted in an excretion of urinary nitrogen amounting to 0.496 gm. per hour, produced, in the earlier group of experiments, an increase in metabolism of 39.4 per cent. In the present experiments the administration of gelatin which caused a nitrogen excretion of 0.430 gm. per hour, raised the heat production 38.5 per cent.²

² It is interesting to compare the present basal metabolism of this dog, weighing approximately 6.5 kilos, with the one studied by Plummer, Deuel, and Lusk (9). Their animal, whose weight varied between 6.3 and 7.0 kilos, had a basal metabolism, if all six experiments of the first series be averaged, of 12.68 calories, practically identical with that obtained from our dog.

TABLE II
Administration of Gelatin, Casein, and the Fractions of These Proteins Extracted by and Precipitated in Butyl Alcohol
(Fraction I)

Average per hour of 4 hour period

Food	Experi- ment No	Date	Urinary N	O ₂	R Q	Calories	Increase over basal	Remarks
Gelatin (6 gm. N)	33	Oct 28	gm	gm			per cent	
	48	Nov 27	0 448 0 411	5 36 5 21	0 86 0 86	17 38 16 96	40 2 36 8	
	Average						38 5	
Fraction I of gelatin (5 gm. N).	40	Nov 10	0 340	5 35	0 89	17 71	42 8	
	34	Oct 30	0 319	4 87	0 86	16 00	29 0	
	46	Nov 22	0 300	4 82	0 85	15 71	26 7	
Gelatin (3 gm. N) and Fraction I of gelatin (1 5 gm. N).	Average						27 9	
	35	Nov 1	0 408	5 06	0 87	16 48	32 9	
	45	" 19	0 401	5 10	0 86	16 61	34 0	
Casein (6 gm. N)	Average						33 5	
	68	Jan 21	0 254	5 01	0 85	16 53	33 3	
	43	Nov 16	0 416	5 86	0 80	18 87	52 2	Exp 42, discontinued after the 2nd hr, gave an average increase for these 2 hrs. of 45.8 per cent.
Fraction I of casein (5 gm. N).	49	" 29	0 461	6 00	0 77	19 13	54 3	
	Average						53 3	

Fraction I of casein (3 gm. N).	54	Dec. 9	0 295	4 99	0 83	16 32	31 6
	56	" 13	0 348	5 01	0 83	16 28	31 3
	Average						31 5
	. . .						
Casein (3 gm N) and Fraction I of casein (1.5 gm. N).	58	Dec. 20	0 336	4 75	0 83	15 62	26 0
	70	Jan 26	0 235	4 71	0 83	15 49	24 9
	Average						25 0
	..						

There was considerable fluctuation in the R.Q., which varied from 0.80 to 0.89, the oxygen consumption being even more constant than the heat production.

B. Gelatin and Fraction I³ of Gelatin Hydrolysate.

As has already been mentioned, gelatin containing 6 gm of nitrogen, when fed to a dog with about 150 cc of water, increased the metabolism in two experiments, 40.2 per cent and 36.8 per cent respectively, an average of 38.5 per cent (Table II). In experiments reported previously (4), it has been found that the gelatin hydrolysates had approximately the same effect.

Fraction I of the gelatin hydrolysate, according to Dakin (5), contains glycine, alanine, serine, phenylalanine, tyrosine, and hydroxyproline. We found the total nitrogen to be 14.1 per cent, compared with the 13.7 per cent found by Dakin. When this fraction was administered, in a quantity containing 5 gm. of nitrogen, the heat production rose to 17.7 calories, an increase of 42.8 per cent over the basal level. This stimulating effect upon cellular metabolism, while somewhat greater per gm of nitrogen than that produced by gelatin itself or its hydrolysate, is not markedly so. Assuming that the cause of the specific dynamic action of gelatin lies in this fraction, the result is at first glance somewhat surprising, for one would suppose that per gm of nitrogen, Fraction I would have a considerably greater effect upon heat production than would the entire protein, since the latter contains other amino acids which we have no present reason for believing have any marked specific dynamic action of their own. The discrepancy is probably in part due to the fact, mentioned above, that of the 25.5 per cent of glycine in the gelatin molecule only 3.8 per cent (Dakin) is extracted by butyl alcohol, the remaining 21.7 per cent remaining behind in the aqueous residue, and being separated finally from the dicarboxylic acids.

When 3 gm of Fraction I of the gelatin hydrolysate were given to the dog, the resulting rise of metabolism amounted, in two experiments, to 29.0 and 26.7 per cent respectively; an average increase of 27.9 per cent (Table II). When this is compared with

³ Where Fraction I is hereafter referred to in the text, it will be understood to mean the fraction extracted by and precipitated in butyl alcohol.

the 42.8 per cent rise seen when 5 gm. of this fraction were administered, it is clear that the specific dynamic action of the fraction is roughly proportional to the amount of the substance ingested by the animal. In this respect it behaves in a manner similar to that of the proteins, whose specific dynamic action increases as the amount metabolized increases (Williams, Riche, and Lusk (10), Weiss and Rapport (3)); and also to that of the amino acids, glycine and alanine, as Lusk (7) has shown.

Gelatin containing 6 gm. of nitrogen had increased the heat production 38.5 per cent, and Fraction I of the hydrolysate, containing 3 gm. of nitrogen, had raised the metabolism 27.9 per cent. If, when half portions of these two substances—that is, gelatin (3 gm. of N) and Fraction I (1.5 gm. of N)—were given together, their specific dynamic actions were summated, one would expect a theoretical rise in the heat production of approximately 33 per cent. This experiment was performed twice, with almost identical results. In one case the increase over the basal level was 32.9 per cent, in the other 34.0 per cent, an average of 33.5 per cent. When gelatin is given with the monoamino, monocarboxylic acid fraction of its hydrolysate, the specific dynamic actions of the two substances are summated.

C. Casein and Fraction I of Casein.

The specific dynamic effect of casein (6 gm. of N), in the one experiment done, proved to be 33.3 per cent (Table II). When Fraction I (5 gm. of N) of the hydrolysate of this protein was given to the dog the resulting rise in metabolism was very large, in one case amounting to 52.2 per cent over the basal, in another to 54.3 per cent, an average of 53.3 per cent. (In a third experiment the animal vomited during the 3rd experimental hour. During the first 2 hours, when the recording apparatus indicated no evidence of undue movement, the metabolism showed an average increase of 45.8 per cent.) This remarkably large effect, actually greater than that of the gelatin fraction, was noteworthy in view of the negligible amount of glycine, and the almost equally small amount of alanine, contained in the fraction.

On two occasions an amount of Fraction I containing 3 gm. of nitrogen was administered. Again almost identical results were obtained, the animal appearing to react to the foodstuff with

almost the quantitative exactness of a solution in a test-tube. In one experiment the increase in metabolism was 31.6 per cent; in the other 31.3 per cent. As in the case of Fraction I of the gelatin hydrolysis the rise in heat production paralleled the amount given. *The specific dynamic action of the monoamino, monocarboxylic acid fraction of a protein hydrolysate is proportional to the amount ingested.*

The possibility of summation of casein with this fraction of its digestion products ~~was tested~~. A theoretical summation of half portions of these two substances would have resulted in an increase of 32 per cent; a complete absence of summation in an increase of 16 per cent. The actual rise found, in two experiments, amounted to 26.0 and 24.9 per cent, an average of 25 per cent. This was not complete summation, it is true, and we are unable to account for the slight discrepancy between this result and that obtained when gelatin and Fraction I of the gelatin hydrolysis were given together. On the other hand, it is equally true that the experiments did not show complete inhibition of the stimulating properties of either of the substances administered. While the result is not clean cut, it would appear probable that when casein and Fraction I of casein hydrolysate are given together, the specific dynamic action of neither is nullified.

D. Glycine and Fraction I of the Gelatin and Casein Hydrolysates.

10 gm. of glycine caused a rise in the heat production of 29.2 per cent (Table III). In the same animal, 8 months previously, the increase due to the same amount of the material was 27.7 per cent. In the dog studied by Plummer, Deuel, and Lusk (9), of approximately the same weight and basal metabolism, the specific dynamic action of this quantity of glycine in the first series of experiments was practically identical with that found in our animal, namely 28 per cent.

15 gm. of glycine produced an increase of 34.9 per cent. It will be recalled that the rise in metabolism due to Fraction I of the gelatin hydrolysis, containing 3 gm. of nitrogen, was 27.9 per cent. When half portions of each of these substances were given together, the resulting increase of heat production was 32.7 per cent. This is slightly greater than the sum of the expected rise in metabolism of each of these substances, which would have been about 29 per cent.

TABLE III.
Administration of Glycine and the Fractions of Gelatin and Casein Extracted by and Precipitated in Butyl Alcohol.
(Fraction I)
 Average per hour of 4 hour period

Food	Experiment No	Date	Urinary N gm	O ₂ gm	R Q	Calories	Increase over basal per cent	Remarks
Glycine, 10 gm	65	Jan 15	0 256	4 69	0 94	16 02	29 2	3 hrs.
" 15 "	66	" 17	0 265	5 00	0 91	16 73	34 9	
Fraction I of gelatin (3 gm. N)	Average of Experiments 34 and 46.		0 310	4 85	0 86	15 86	27.9	
Glycine, 7.5 gm and Fraction I of gelatin (1.5 gm. N)	52	Dec. 4	0 379	4 85	0 99	16 46	32 7	
Fraction I of casein (3 gm N)	Average of Experiments 54 and 56.		0 322	5 00	0 83	16 30	31 5	
Glycine, 7.5 gm. and Fraction I of casein (1.5 gm. N)	53	Dec. 6	0 362	4 97	0 93	16 71	34 8	
	55	" 11	0 351	4 93	0 93	16 59	33 8	
					Average.		34 3	

When 7.5 gm. of glycine and Fraction I of casein hydrolysate containing 1.5 gm of nitrogen—that is, half portions of each—were administered together, the expected rise of oxygen consumption and heat production, provided summation occurred, was about 30 per cent, the actual rise found, in two experiments, was 34.8 and 33.8 per cent, an average of 34.3 per cent.

It is clear that *when glycine is given together with the mono-amino, monocarboxylic acid fraction of a protein hydrolysate, the specific dynamic actions of the two substances are summated.*

For the sake of clearness, a table of the effects of combining proteins with their split-products, as thus far studied, is given, as follows.

Protein	given	with	protein	results	in	a	summation	of	effect.
"	"	"	hydrolysate	"	"	"	"	"	"
Glycine	"	"	glycine	"	"	"	"	"	"
Protein	"	"	"	"	"	"	nullification	of	the glycine effect
Hydrolysate	given	with	glycine	results	in	a	nullification	of	the glycine effect
Fraction I	given	with	protein	results	in	a	summation	of	effect
"	"	"	glycine	"	"	"	"	"	"

The implications of this summary are not at all clear. It is obvious that proteins and their hydrolysates behave similarly. It appears equally evident that when proteins and hydrolysates are given with glycine, something in the protein molecule, even when it is hydrolyzed, is capable of masking the effect of the glycine. It has been postulated by Weiss and Rapport (3) that this something is perhaps a polypeptide formed by the combination of glycine with one or more amino acids. When glycine itself is given in increasing quantities, the specific dynamic action is proportional to the amount metabolized. Recently, Plummer, Deuel, and Lusk (9) have studied the effect of glycyl-glycine, and have found that it is in all respects similar, in equivalent quantities, to that of glycine. Hence one of the possible combinations, namely that of glycine with itself, is ruled out. Similarly, Weiss and Rapport found that asparagine did not nullify the action of glycine. From the experiments reported in this paper, it becomes evident that whatever be the cause, in the protein molecule, resulting in an inhibition of the specific dynamic action of glycine, that

cause does not lie in Fraction I. It is possible that a study of the other fractions may throw some light on this subject. It would be easy to become involved in a maze of speculation in regard to these phenomena, particularly in view of the fact that in respect to its behavior when given with a protein, Fraction I acts like the protein itself, and not like glycine. In the absence of more coherent data, however, such speculation would at present be fruitless.

E. Alcohol Checks.

In the course of this work six alcohol checks were done (Table IV). The average respiratory quotient was 0.663, with a mean error of 0.6 per cent. The maximum error in any one determination was 1.9 per cent.

TABLE IV
Alcohol Checks

Date	Experiment No	Calories	R Q
<i>1926</i>			
Oct 23	11	12 17	0.667
" 26	12	11 56	0.657
Nov 6	13	11 14	0.664
" 30	14	11 30	0.666
Dec 15	15	21 87	0.670
<i>1927</i>			
Jan 27	16	11 58	0.654
Average			0.663

Mean error = 0.6 per cent

Maximum error = 1.9 per cent

SUMMARY.

1. The fraction of gelatin hydrolysate extracted by and precipitated in butyl alcohol, designated for convenience as Fraction I, and containing practically all of the monoamino, monocarboxylic acids, has a slightly greater specific dynamic action, per gm of nitrogen, than has gelatin, in spite of the fact that most of the glycine of the protein is not present in this fraction.

2. Fraction I of the casein hydrolysate, in equivalent quantities, has a 60 per cent greater effect on the metabolism than has casein itself.

3. The specific dynamic effect of Fraction I of the casein hydrolysate is somewhat greater than the corresponding fraction of gelatin.

4. The specific dynamic action of Fraction I of both gelatin and casein hydrolysates is proportional to the amount ingested.

5. When Fraction I of the gelatin hydrolysate is administered with gelatin, the specific dynamic actions of these two foodstuffs are summated. This summation is not entirely complete when casein and Fraction I of the casein hydrolysate are given together.

6. When Fraction I of the hydrolysis of either casein or gelatin is fed to a dog along with glycine, the effect of the glycine is not nullified. In this respect the fraction differs from its parent protein, and from the hydrolysate of the protein.

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THE EFFECTS OF PROTEIN SPLIT-PRODUCTS UPON METABOLISM.

II. THE INDIVIDUAL AMINO ACIDS OF FRACTION I OF THE BUTYL ALCOHOL EXTRACTION, AND THEIR RELATION TO THE SPECIFIC DYNAMIC ACTION OF PROTEIN.

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INTRODUCTION.

In the preceding paper of this series (1), we have detailed certain observations on the fractions of gelatin and of casein hydrolysates extracted by and precipitated in butyl alcohol (for convenience we have designated this as Fraction I). In the experiments described in that paper, we found that Fraction I of casein hydrolysate had a specific dynamic action which was, per gm. of nitrogen ingested, slightly greater than that of this fraction of gelatin; and that Fraction I of the hydrolysis of casein had an effect on metabolism that was fully 60 per cent greater than an equivalent amount of the protein itself. If the initial assumption be made that the specific dynamic effect of the proteins is due to amino acids in this fraction, the resulting large rise in metabolism upon the ingestion of these fractions is explicable. Moreover, in the case of gelatin, this result is plausible at first glance on the theory, advanced by Lusk (2), that the specific dynamic action of proteins is due to the massed action of certain metabolic products of the individual amino acids, and that the major portion of the effect is due to glycine and alanine, which Lusk (3) proved to have a powerful effect on the heat production. But the theory in addition to its failure to explain certain differences between the dynamic effects of proteins on the one hand, and of glycine and alanine on the other, which have been discussed in the preceding paper, also does

not explain why casein causes practically as great a heat production as gelatin. This discrepancy resulted in the postulated hypothesis (4) that the specific dynamic action of proteins might in part, at least, be due to the resynthesis of certain amino acids into more complex forms having a dynamic effect which was different from that of the amino acids themselves. Analyzing the results of the study on Fraction I, it is apparent that this discrepancy persists, for, in the first place, Fraction I of casein hydrolysis has a greater absolute effect than the same fraction of gelatin, in the second place it has a greater relative effect, compared with its parent protein, than has the corresponding fraction of gelatin. Yet the amount of glycine and alanine in this fraction in the case of casein is very small, whereas these acids constitute a relatively large percentage of Fraction I in gelatin.

In undertaking the experiments described in the present paper, therefore, we were confronted by two major questions: (1) Can the metabolism of other amino acids than glycine and alanine produce sufficient effect on the cellular oxidation in the organism to account for the specific dynamic action of proteins? (2) Is it necessary to return to a modification of the old and apparently outworn theory of the resynthesis of amino acids into more complex forms in the course of their catabolism?

In answer to the questions posed above, the evidence to be adduced in the following communication will attempt to show (1) that in the case of Fraction I the dynamic effect of the whole fraction can be explained by the additive effects of its constituent amino acids, (2) that while in the case of gelatin this effect is largely due to glycine and alanine, this is by no means true in casein and other proteins, (3) that phenylalanine per gm. ingested has a more powerful effect on the metabolism of the organism than has an equivalent amount of glycine, (4) that the additive effects of those acids known to have a specific dynamic action do not suffice to explain the specific dynamic action of certain proteins.

Technique and Methods of Calculation.

The method of determining the metabolism has been described in the preceding paper, as has the preparation of Fraction I of the protein hydrolysates. In making the calculations of the heat production after giving individual amino acids, a question arose

similar to the one with which we were confronted in calculating the protein hydrolysate and Fraction I experiments. The caloric values per gm. of urinary nitrogen, and the corresponding values of the respiratory CO_2 and O_2 , are different for each amino acid. Because of these different values, the relative values of protein (including protein fractions and amino acids) and non-protein heat production and R.Q. are considerably altered, depending upon whether the experiment is calculated upon the factors for meat protein, commonly employed, or upon the special factors for the particular substance administered. In each case, we are confronted by an uncontrolled variable, for the urinary nitrogen, over a short period of time, is admittedly only an approximate index of the amount of the ingested substance metabolized. It would, moreover, be necessary to employ the special factors only in case the calculation of the total metabolism with which we are at present particularly concerned, were markedly different depending upon which factors are employed. We will discuss this at somewhat greater length in considering the metabolism of phenylalanine. Suffice it to say, for the moment, that the total heat production is not markedly different, and we have therefore been content to follow the routine established hitherto, and to calculate the metabolism on the usual factors, involving as it does an inappreciable error.

EXPERIMENTAL.

A. Basal Metabolism.

The figures for the basal metabolism given in Table I of the preceding paper of this series covered the period of the present research as well, and the same mean figure, 12.40 calories, is accepted as the basal level from which increases could be calculated, as there was no noteworthy deviation for a period of 3 months. (The validity of the results in the present communication is likewise attested by the alcohol checks tabulated in the previous paper.)

B. Amino Acids in Fraction I.

The relative effects of Fraction I of gelatin and casein hydrolysis made it appear advisable to make a detailed investigation of these fractions.

Table I gives the percentage of the total amino acids in Fraction I of both gelatin and casein. The figures for gelatin are those given by Dakin (5), and for casein those compiled from his own work and that of others by Foreman (6). It will be seen that, although in both proteins Fraction I contains almost the same

TABLE I
Amino Acid Content of Fraction I

	Gelatin	Casein
	<i>per cent of total</i>	<i>per cent of total</i>
Glycine*	3.8 (21.7 per cent in aqueous residue)	0.45
Alanine	8.7	1.85
Leucine	7.1	9.7
Isoleucine	None.	None.
Valine	"	7.93
Serine	0.4	0.5
Hydroxyproline†	14.1(?)	0.23
Cystine	None	None
Tryptophane	Destroyed by acid hydrolysis	(1.5 per cent)
Phenylalanine	1.4	3.88
Tyrosine	0.01	4.5 (40 per cent crystallizing out of neutral hydrolysate)
Total	35.6	29.04
Remaining amino acids	= 32.7	59.44
Glycine in aqueous residue	= 21.7	Tryptophane = 1.5
Total amino acids	= 90.0	89.98

* Only partially extracted by butyl alcohol

† A small percentage of the hydroxyproline is present in other fractions.

percentage of the total amino acids, a relatively far larger amount of glycine (even though most of the glycine remains unextracted) and of alanine is contained in this fraction of the gelatin hydrolysate than in that of casein. Of the remaining acids, gelatin contains chiefly leucine and hydroxyproline; casein, chiefly leucine

and valine, with comparatively large amounts of phenylalanine and tyrosine as well

Even assuming glycine and alanine to play a predominant rôle in the causation of the specific dynamic action of Fraction I of the hydrolysis of gelatin, it is clear that they could not occupy the same important position, quantitatively, in Fraction I of the casein hydrolysate, unless the assumption is made that in the case of the latter the ingestion of the fraction results in a synthesis of these acids. The evidence that has been obtained by Csonka (7) on the lack of glycine synthesis following the ingestion of casein, and to which reference has been made in a previous paper (4), indicates that this possibility can be ruled out.

What then, is the cause of the specific dynamic action of Fraction I of the hydrolysis of casein? It appeared that a systematic consideration of the individual amino acids constituting Fraction I in both of these proteins would be the most feasible way of attacking this problem. In the following consideration of the individual acids it must be borne in mind that in the case of casein Fraction I has a greater effect than the corresponding fraction of the gelatin hydrolysate (53.3 per cent increase as opposed to 42.8 per cent).

C. Individual Amino Acids Other than Glycine and Alanine.

1. Leucine.

Lusk (8) has given 20 gm. of leucine to a dog weighing 9.5 kilos, with a resulting increase of metabolism of only 10 per cent, in spite of the fact that the urinary nitrogen indicated a considerable breakdown of the ingested material. We have not repeated this experiment, but on the basis of comparisons with the increases obtained from different amino acids by Lusk and his coworkers and by ourselves on this and other dogs, we have estimated that if our dog had been given 10 gm. of leucine, the rise in the heat production would have approximated, and certainly would not have greatly exceeded, 10 per cent. If anything, this figure may be somewhat too high. At all events, it is clear that leucine alone, being present in only very slightly greater concentration in casein than in gelatin (9.7 per cent as opposed to 7.1 per cent), could not account for the specific dynamic action of Fraction I.

2. Isoleucine, Cystine, and Tryptophane.

These may be very briefly dismissed. Isoleucine is present in neither fraction, tryptophane is destroyed by the acid hydrolysis preceding the butyl alcohol extraction, cystine is absent from gelatin and is present in only questionable traces in casein. Moreover, it has been found (9) that the addition of 1 gm. of cystine to gelatin did not cause an increase of metabolism greater than that caused by gelatin alone, when given to a dog.

3. Serine and Hydroxyproline.

Serine is present in practically the same small concentrations in gelatin and casein. If, therefore, serine were the cause of the specific dynamic action of casein, the serine effect should also have been present in gelatin, and would have to be added to the known effect, in the latter protein, of glycine and alanine. On this basis, Fraction I in the case of gelatin, would have produced at least twice as great an increase in the metabolism as did Fraction I of the casein hydrolysate. But in point of fact the rise of heat production after giving the latter was, as has been mentioned, actually greater than after giving the gelatin fraction. Serine, therefore, is excluded as an important factor.

Hydroxyproline is present in large concentration in gelatin, 14.1 per cent. But it is almost completely absent in casein and can therefore be ruled out.

4. Valine

The possibility existed that valine, which is lacking in gelatin but is present in the large concentration of 7.93 per cent in casein, might be the cause of the specific dynamic action of the casein fraction. But when we gave 10 gm. of *dl*-valine (Eastman) to the dog (Table II and Chart I), there was no increase whatsoever over the basal metabolism. Thus, the average basal level was 12.40 calories, with a maximum deviation of 3.8 per cent. On giving valine, the average metabolism of 3 hours (up to the 5th hour after ingestion of the foodstuff) was 12.77 calories, a heat production that was the same as the basal within the limits of experimental error. Nevertheless the urinary nitrogen excretion was 0.213 gm. per hour, more than double the basal nitrogen,

TABLE II.
Administration of Valine

Food	Experiment No.	Date	Time	Urin- ary N	O ₂	R Q	Calo- ries	In- crease over basal	Remarks.
Valine, 10 gm in 150 cc water at 9 05 a m.	57	Dec 16		<i>gm</i>	<i>gm</i>			<i>per cent</i>	
			10 05-11 05	0 213	3 78	0 87	12 53		4th hr not calculated as animal was restless.
			11 05-12 05	0 213	3 75	0 88	12 50		
			12 05- 1 05	0 213	4 03	0 85	13 29		
			Average	0 213	3 85	0 86	12 77	3 0	

indicating the combustion of over 5 gm , or more than half, of the ingested amino acid. *Valine* has no specific dynamic action. It follows therefore that, acting individually, it can have no causative relation to the specific dynamic action of casein.

IR Q	0.94	0.93	0.96	0.89
CALORIES	16.02	12.77	17.29	14.70
% INCREASE OVER BASAL	29.2	3.0	39.4	18.6

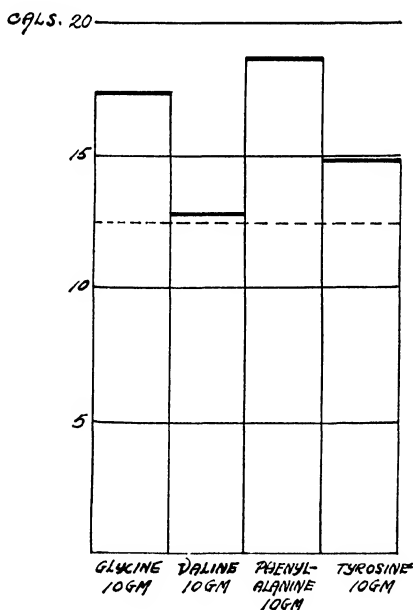


CHART I. The effect on the metabolism of ingesting glycine, valine, phenylalanine, and leucine. The dash line shows the basal level.

5. Phenylalanine.

So far we had excluded all of the amino acids of the first fraction of the casein hydrolysate except phenylalanine and tyrosine as playing a predominant rôle in the specific dynamic action of casein. In view of the fact that Lusk had found tyrosine not to have a great specific dynamic action per gm. ingested, and in view also of the generally accepted similarity in the course of the oxidation

TABLE III.
Administration of Phenylalanine.

Food.	Experiment No	Date	Time.	Urinary N	O ₂	R Q	Calories	Increase over basal.	Remarks
				gm	gm			per cent	
Phenylalanine, 10 gm. at 10 a.m.	62	Dec. 31	11 00-12 00	0 203	4 65	1 01	15 73		Calculated on phenylalanine factors, increase = 37 per cent (see text).
			12 00- 1 00	0 203	4 97	0 99	16 86		
			1 00- 2 00	0 203	5 27	1 01	17 92		
			2 00- 3 00	0 203	5 47	1 01	18 63		
			Average	0 203	5 09	1 01	17 29	39 4	
Phenylalanine, 8 5 gm. at 9.15 a.m.	64	Jan. 13	10 15-11 15	0 227	4 64	0 95	15 87		
			11 15-12 15	0 227	4 81	0 89	16 24		
			12 15- 1 15	0 227	4 96	0 92	16 87.		
			1 15- 2 15	0 227	4 99	0 92	17 06		
			Average.	0 227	4 85	0 92	16 51	33 1	
Average of Experiments 62 and 64				0 215	4 97	0 96	16 90	36 3	For details of two other experiments performed with phenylalanine, see text.

of tyrosine and phenylalanine, we did not expect to obtain a very large effect from the latter acid. However, when we administered *dl*-phenylalanine (Pfanstiehl), we observed a very marked specific dynamic action. We repeated this experiment four times. On the first two occasions, we made a suspension of 10 gm. of the amino acid in water, and attempted to give it by stomach tube. On both occasions we encountered difficulties because of the rapid settling of the suspension and clogging of the tube. As a result of this we lost in both instances an indeterminate amount of the material. We are unable to state positively, therefore, just how much of the 10 gm. was actually introduced into the animal. In both of these experiments the metabolism was determined for only a 3 hour period, as the dog became restless during the 4th hour. In one experiment the average rise in heat production was 24.7 per cent, with a nitrogen excretion of 0.183 gm. per hour. In the other, the increase was 23.5 per cent. The urine was not obtained in this experiment, as the large amount of water used in attempting to wash out the stomach tube caused the dog to void during the 4th hour.

These experiments, while not entirely satisfactory, established the fact that phenylalanine had a considerable specific dynamic action. The importance of the result warranted further investigation, and by varying the method of administration of the substance, two satisfactory experiments were obtained. The phenylalanine was made into a pasty ball with a small amount of water, and this paste was introduced into the back of the mouth and swallowed by the animal. In this way practically none of the material was lost. In one experiment (Table III), 10 gm. of phenylalanine resulted in an increased metabolism of 39.4 per cent, with an excretion of urinary nitrogen of 0.227 gm. per hour (or 0.127 gm. per hour above the basal nitrogen). In another experiment we were unable to give more than 8.5 gm. of phenylalanine, as we had no more of this rather expensive material at hand. The heat production rose 33.1 per cent, and the urinary nitrogen was 0.203 gm. per hour. Assuming that the effect is proportional to the amount ingested, the rise induced by 10 gm. of the material would have been 38.9 per cent. The figure obtained in the earlier experiment can, therefore, be accepted as substantially correct.

It is evident that *phenylalanine has a very powerful specific*

dynamic action. If its effect in raising the heat production be compared with that of glycine, it is also apparent that, per gm. of substance ingested, *it is more effective in raising the metabolism than glycine itself*, for 10 gm. of the latter, when administered, raised the metabolism of this dog 29.2 per cent.¹

In a recent paper, Seth and Luck (11) have denied the view of Lusk "that the specific dynamic action of amino acids is due to a chemical stimulation of the body cells to a higher level of metabolism by some intermediary metabolites of the amino acids other than the immediate products of their deamination." Apart from an argument (p. 375), based on an apparent misconception of the "extra glucose" method of calculating the rate of amino acid

¹ It would not be out of place here to refer to the method of calculating the phenylalanine experiments. As given in Table III, the heat production is calculated as though the urinary nitrogen were due entirely to meat protein. This is of course erroneous, and we have deemed it advisable to investigate the possible extent of the error in computing the total metabolism. The following calculation may be made

Heat of combustion of 1 gm phenylalanine	=	6 752*	calories
" " " " 1 mol "	=	1 114	
" " " " 1 gm urea	=	2 528†	
1 gm phenylalanine yields 0.181 gm urea	=	0.457	
Physiological value of 1 gm phenylalanine	=	6 295	
1 gm phenylalanine	=	2.267 gm respiratory CO ₂	
	=	1.939 " " O ₂	
R Q	=	0.85	
1 gm phenylalanine N	=	26.33 gm respiratory CO ₂	
	=	22.53 " " O ₂	
	=	73.11 calories	
Value of 1 liter O ₂	=	4 636 calories	

Using these figures, and calculating the metabolism, the rise in heat production following the ingestion of 10 gm of phenylalanine becomes 37 per cent, instead of the 39.4 per cent given in the table. But on the other hand, the urinary nitrogen is an inexact method of estimating the amount of phenylalanine metabolized, hence this method also involves an error, which may be greater than the slight one noted, and is probably in the opposite direction. Taking these facts into consideration, we have used the usual factors in all computations except those for glycine, where the work of Csonka (10) has given us a more nearly exact index of the amount of amino acid metabolized.

* Fischer, E., and Wrede, F, *Sitzungsber. k. preuss. Akad. Wissensch.*, 1904.

† Emery, A. G., and Benedict, F. G., *Am. J. Physiol.*, 1911, xxviii, 301

metabolism, and implying that Lusk regards the glucogenetic properties of an amino acid as the cause of its specific dynamic action, these authors rest their challenge of Lusk's views chiefly on facts ascertained by themselves and by Bang (12), that the amino nitrogen content of the blood reaches and remains at a higher level after glycine and alanine ingestion than after the ingestion of other amino acids studied. Correlating this with some of the then known data, they therefore conclude that, "The specific dynamic action of an amino-acid is proportional to its power of increasing the amino-N content of the blood, following the gastro-intestinal absorption of the amino-acid."

Considering the powerful specific dynamic action of phenylalanine, as shown in the present experiments, this conception ceases to be tenable. As proved by Levene and Kober (13) and Levene and Meyer (14), the metabolism of phenylalanine is slower in its course than that of either glycine or alanine, and corresponds very nearly to that of leucine, which Seth and Luck have themselves shown to increase the blood amino N very little. Yet phenylalanine, as we have found, has a more profound effect on the metabolism per gm ingested, and an even greater effect per gm. metabolized than has glycine.

6. Tyrosine.

Lusk (3) had found that 20 gm of tyrosine, given to a dog weighing 9.7 kilos, increased the metabolism 10 per cent (13.5 per cent on the oxygen). In view of the conspicuous effect of phenylalanine, we decided to repeat this experiment on our dog, which was smaller than the one used by Lusk. We employed the method of administering the substance—namely of making a thick paste with water and placing it in the back of the mouth—that had proved effectual in the case of phenylalanine. In two experiments, the ingestion of 10 gm of tyrosine provoked increases in the heat production of 16.0 per cent and 21.1 per cent, respectively (Table IV). This is somewhat higher than the result obtained by Lusk, even when the difference between the size of the animals is taken into consideration, and makes the specific dynamic action of tyrosine, per gm. ingested, appear to be comparable with that of alanine. Folin and Denis (15) upon injecting tyrosine into the

intestine of cats, could find no evidence of absorption of the substance by studying the non-protein and urea nitrogen of the blood, and only by qualitative colorimetric test were they able to demonstrate any sign of absorption. Lusk, in the experiment quoted above, found no increase in urinary nitrogen that he could not ascribe to possible experimental error. The basal nitrogen in our experiments averaged 0.1 gm. per hour, the variations being from 0.098 to 0.107 gm. Upon giving tyrosine, the average

TABLE IV
Administration of Tyrosine

Food	Experiment No	Date	Time	Urinary N	O ₂	R Q	Calories	Increase over basal
				gm	gm			per cent
Tyrosine, 10 gm at 9 10 a m	63	Jan. 10	10 10-11 10	0 139	4 52	0 86	15 15	
			11 10-12 10	0 139	4 52	0 86	15 15	
			12 10- 1 10	0 139	4 46	0 86	14 94	
			1 10- 2 10	0 139	4 42	0 86	14 82	
			Average	0 139	4 48	0 86	15 02	21 1
Tyrosine, 10 gm at 9 35 a m	67	Jan 19	10 34-11 34	0 135	4 48	0 91	15 23	
			11 34-12 34	0 135	4 39	0 92	14 93	
			12 34- 1 34	0 135	4 13	0 89	13 95	
			1 34- 2 34	0 135	3 95	0 89	13 41	
			Average	0 135	4 24	0 91	14 38	16 0
Average								18 6

nitrogen elimination, in the two experiments, was 0.135 and 0.139 gm. per hour, the increase over the basal being still small, it is true, but unmistakable. It is probable that the difference in the specific dynamic action of this amino acid, as observed by Lusk and by us, was due to the fact that there was, in our animal, a greater absorption and metabolism of the substance. - On the basis of the nitrogen excretion, 1.9 gm. of the tyrosine we administered were metabolized. While the evidence indicates that phenylalanine is more slowly broken down in the organism than

are the lower amino acids, it appears, nevertheless, on the basis of the urinary nitrogen in our experiments, to be more rapidly metabolized after ingestion than is tyrosine

Assuming Csonka's figures for the metabolism of glycine to be approximately correct, and using the admittedly inexact criterion (over this period of time) of the urinary nitrogen as an index of the metabolic breakdown of phenylalanine and tyrosine, it may be said that, per gm. of substance metabolized, phenylalanine is $1\frac{1}{2}$ times, and tyrosine $2\frac{1}{2}$ times, as powerful in stimulating oxidations, as is glycine. This is necessarily only a rough approximation

In view of the possible relationship of the aromatic acids to the internal secretions of the thyroid and the adrenal medulla, the observed effects of phenylalanine and of tyrosine upon metabolism induce interesting speculations. If these two acids be indeed precursors of thyroxin and epinephrine, in what way, for example, is the stimulating potential of the former raised in the course of their elaboration to the hormones?

DISCUSSION.

A Specific Dynamic Action of Fraction I.

We have thus far made a systematic survey of the individual amino acids in Fraction I, with a view primarily to determine whether the specific dynamic action of this fraction of the hydrolysis of casein could be thus explained. In the course of the survey, we have determined the effect of those amino acids whose stimulating power it seemed necessary to establish in order to arrive at a proper conclusion in this connection. These observations brought out the fact that of all the amino acids in this fraction, phenylalanine has the most powerful influence upon the heat production per gm. ingested. It is likewise clear that tyrosine, when absorbed, has also a profound effect.

Applying the observed data to a consideration of the specific dynamic action of Fraction I, certain interesting facts become evident. Of the known amino acids in the protein molecule, five have thus far been proved to have a specific dynamic effect. These five are glycine, alanine, leucine, phenylalanine, and tyrosine. They all lie in Fraction I. Certain other acids, such as

glutamic acid, aspartic acid, and valine, as well as cystine (at least under certain conditions) have no effect whatever. The remaining acids have not been individually studied

TABLE V.

Theoretical Effect of Amino Acids Contained in Fraction I and Known to Have a Specific Dynamic Action, Compared with Effect of Entire Fraction

Amino acids	Effect actually found		Gelatin Fraction I (35.45 gm = 5 gm N)				Casein Fraction I (43.86 gm = 5 gm N)			
	Amount ingested	Increase over basal	Amount in Fraction I		Theoretical increase over basal		Amount in Fraction I		Theoretical increase over basal	
	gm	per cent	per cent	gm	per cent	per cent	gm	per cent		
Glycine	10	29	10.7*	3.79	11.0	1.6	0.7	2.0		
Alanine	10	20†	21.6	7.66	15.3	6.4	2.81	5.6		
Leucine	10	10‡	19.9	7.05	7.1	33.4	14.65	14.7		
Phenylalanine	10	39	3.9	1.38	5.5	13.4	5.88	23.2		
Tyrosine	10	21	Trace	Trace		6.9§	3.02	6.3		
Total					38.9			51.8		
Found on administering entire fraction					42.8			53.3		

* Only partially extracted by butyl alcohol

† Computed from the data of Lusk (3, 8) and of Weiss and Rapport (4)

‡ Computed from the data of Lusk (3). The figure is based on the administration by Lusk of 20 gm of the substance to a larger dog than the one used in these experiments, and is obtained by a comparison of the effects of leucine and of the other amino acids given in the experiments of Lusk and in those of ourselves. If anything, it probably errs in being too large.

§ 40 per cent of tyrosine crystallizes out of neutral hydrolysate (Dakin (5)).

The calculations in this table, as well as in Tables VI and VII are to be regarded merely as approximations. The computations of the theoretical effects of the amino acids contained in Fraction I are based on the assumption that the specific dynamic action is in each case proportional to the amount of the amino acid. In each case, the highest trustworthy figures obtained for each amino acid are given.

Of the five acids known to have a specific dynamic action, the effect of a known quantity of these—namely 10 gm. each of glycine, phenylalanine, and tyrosine—has been ascertained in this animal.

The approximate effect of the other two, namely alanine and leucine, in the same amount, has been estimated from the data of Lusk and of Weiss and Rapport. Knowing the percentage of these substances in Fraction I, and assuming the specific dynamic action of each to be proportional to the amount ingested, the theoretical rise in heat production that should occur after the ingestion of the amount of each acid contained in the fraction can be calculated. This has been summarized in Table V.

In the case of Fraction I of the gelatin hydrolysate, the effects of the five acids, if summated, would theoretically result in an increased heat production of 38.9 per cent. When the entire fraction was given to the dog, the ensuing rise of metabolism actually found amounted to 42.8 per cent. These results are of the same order of magnitude, and in fact agree within the limit of experimental error, though the latter may be sheer chance. Considering Fraction I of the hydrolysis of casein, we find the same thing to be true. The theoretical summated effects of the five acids would have produced an increased metabolism of 51.8 per cent; the actual rise found when a corresponding quantity of the whole fraction was given was 53.3 per cent. While it is obvious that these calculations cannot be regarded as better than approximations, the fact that the heat production when the whole fraction was ingested was of the same order of magnitude as the summated theoretical effects of the five acids enumerated cannot be ignored. It appears that in the case of both gelatin and casein, *the specific dynamic action of Fraction I can be accounted for by the massed action of glycine, alanine, leucine, phenylalanine, and tyrosine.* It is nevertheless equally clear, that while, in the case of gelatin, two-thirds of the specific dynamic action of Fraction I is provided for by glycine and alanine, these acids have an almost negligible effect in the case of the casein hydrolysate. In the latter substance they account for a mere 15 per cent of the total, the bulk of the effect being provided by phenylalanine (which in itself is responsible for 50 per cent of the whole), leucine, and tyrosine.

B. Specific Dynamic Action of Proteins.

When similar calculations are made in relation to the protein molecule as a whole, still more interesting facts appear. In Table

VI we have made a comparison of the theoretical influence of the amino acids known to have a specific dynamic action with the result obtained on feeding gelatin and casein. In the case of gelatin, the actual rise found on giving the protein was 38.5 per cent, while the theoretical combined effects of the individual acids was 40.4 per cent, of which three-fourths was furnished by glycine. In other words the specific dynamic action of the protein seems to be accounted for by the summated effects of the individual acids. On turning to casein, however, another picture presents itself. Here, of an actual rise in heat production of 33.3 per cent found

TABLE VI *

Theoretical Effect of Amino Acids Known to Have a Specific Dynamic Action Compared with Effect of Gelatin and Casein

Amino acid	Gelatin 37.9 gm (6 gm N)			Casein 44.64 gm (6 gm N)		
	Amount in gelatin		Theoretical increase over basal	Amount in casein		Theoretical increase over basal
	per cent	gm		per cent	gm	per cent
Glycine	25.5	9.7	29.1	0.45	0.2	0.6
Alanine	8.7	3.3	6.6	1.85	0.8	1.6
Leucine	7.1	2.7	2.7	9.7	3.7	3.7
Phenylalanine	1.4	0.5	2.0	3.88	1.7	6.8
Tyrosine	Trace	Trace		4.5	2.0	4.0
Total			40.4			16.7
Found on giving this amount of protein			38.5			33.3

* For explanatory statements, see Table V

on administering the entire protein, only 16.7 per cent could be explained on the basis of the individual acids, a discrepancy of 50 per cent of the total specific dynamic action. This discrepancy is much too large to be the result of errors either experimentally or in the calculations.

We have applied a similar test to two proteins that had been previously studied (9), namely beef and gliadin. These proteins had been given to a dog weighing 11.5 kilos, and if administered in the same amount to the animal, weighing 6.5 kilos, employed in the present research, would probably have caused a greater rise

in the heat production than the figures given in Table VII. This table presents, in the case of these two proteins, a comparison similar to that in Table VI. Considering beef, the combined effects of the individual amino acids are, as in the case of gelatin, apparently able to account for the effect of the whole protein, although the distribution of influence among these acids is decidedly different in the two instances, for the glycine in beef protein has less proportional effect in the molecule than have phenylalanine, alanine, and leucine.

TABLE VII *

Theoretical Effect of Amino Acids Known to Have a Specific Dynamic Action Compared with Effect of Beef and of Gladin

Amino acid	Beef 200 gm (6 gm N) (= 50 gm dry protein)		Gladin 37.5 gm (6 gm N)			
	Amount in beef		Theoretical increase over basal		Amount in gladin	
	per cent	gm	per cent	per cent	gm	Theoretical increase over basal
Glycine	4.0	2.0	6.0			
Alanine	8.1	4.1	8.2	2.0	0.75	1.5
Leucine	14.3	7.2	7.2	6.62	2.5	2.5
Phenylalanine	4.5	2.3	9.2	2.35	0.85	3.4
Tyrosine	4.4	2.2	4.4	1.5	0.56	1.1
Total			35.0			8.5
Found on giving this amount of protein to dog weighing 11.5 kilos (Rapport (9))			33.8			35.3

* For explanatory statements, see Table V

But when we turn to gladin, we have a situation where, as in the case of casein, the individual amino acids are by no means capable of accounting for the observed effect. These acids would result in an increased metabolism of 8.5 per cent; an equivalent amount of gladin actually produced a 35.3 per cent rise. Only one-fourth of the specific dynamic action is accounted for.

It is clear that *although the massed action of the individual amino acids known to have a specific dynamic action is sufficient in the case of some proteins, apparently, to account for the observed*

effects of feeding these proteins to an animal, it is not sufficient, in other proteins, to explain the specific dynamic action of the latter.

What is the reason for this discrepancy? At first glance, there is no obviously large quantity of any one of the amino acids in casein and gliadin to offer a possible explanation. It is true that there are large amounts of glutamic acid in these two proteins, but there is in beef as much glutamic acid as in casein, and Lusk (3), moreover, has shown that this acid is without effect on the metabolism. It seems unlikely, on the basis of the calculations summarized in Table V, that an amino acid of hitherto unsuspected potentialities for stimulating the metabolism should be present in Fraction I. Perhaps a study of the other fractions may reveal such a substance.

SUMMARY.

1. The preceding paper of this series has shown that the fraction of casein hydrolysis extracted by and precipitated in butyl alcohol (Fraction I) has a greater specific dynamic action than an equivalent amount of Fraction I of gelatin hydrolysis. In view of the low glycine and alanine content of casein, a survey is made of the other amino acids in this fraction of both proteins, for the purpose of ascertaining which of the acids may be responsible for the specific dynamic action of Fraction I of the hydrolysis of casein.

2. Of these acids, certain ones may be readily ruled out, as having no predominating influence, as follows: isoleucine, cystine, and hydroxyproline because they are absent from the casein molecule, tryptophane because it is destroyed by the preliminary hydrolysis, serine because it is present in no greater concentration in casein than in gelatin; leucine because it has been shown by Lusk to have an inconsiderable effect, and is not present in Fraction I of casein hydrolysis in sufficiently greater concentration than in gelatin to account for the specific dynamic action of the former

3. Valine is found to have no specific dynamic action whatever.

4. Phenylalanine, however, is found to have a very powerful specific dynamic action—greater than that of glycine, for 10 gm. of the substance produce an increased heat production of almost 40 per cent, as compared with the 30 per cent increase obtained after an equal quantity of glycine is administered.

5. The fact that phenylalanine has a greater effect than glycine upon metabolism renders untenable the theory that the specific dynamic action of an amino acid is proportional to its power of increasing the amino nitrogen content of the blood. It is also clear that the specific dynamic effect of a substance is not proportional to its nitrogen content, for glycine has 18.6 per cent N, whereas phenylalanine has only 8.5 per cent.

6. Tyrosine is found also to have a considerable specific dynamic action, the ingestion of 10 gm. increasing the metabolism 16 to 21 per cent, an effect comparable with that of alanine. The fact that a greater effect is obtained in these experiments than that which Lusk obtained on administering tyrosine, is probably due to greater absorption and metabolism of the substance, as indicated by the urinary nitrogen, in the animal employed in the present research. Per gm. of amino acid metabolized, tyrosine appears to be even more powerful in stimulating oxidation than either phenylalanine or glycine

7. It is calculated that the summated effects of the five amino acids known to have a specific dynamic action (glycine, alanine, leucine, phenylalanine, and tyrosine) are sufficient, theoretically, to account for the effects of Fraction I in the case of both gelatin and casein. While most of the former, however, is accounted for by glycine and alanine, most of the latter is due to phenylalanine and leucine.

8. It is further calculated that while the summated effects of the five amino acids enumerated above are apparently sufficient to account for the stimulating influence on heat production of some proteins, such as meat protein and gelatin, they do not suffice to explain the specific dynamic action of all proteins, since of casein, only 50 per cent, and of gliadin only 25 per cent, of the observed result can be ascribed to the individual acids.

9. It is maintained, on the basis of the present work, that the presence in Fraction I of an amino acid having hitherto unsuspected potentialities for stimulating the metabolism, is unlikely. Study of the other fractions may reveal such an acid.

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STUDIES ON GLUTELINS.

I. THE α - AND β -GLUTELINS OF WHEAT (*TRITICUM VULGARE*).*

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An important criterion used for the characterization of proteins is the amount of certain neutral salts that is required for the precipitation of proteins from their saline or aqueous solution. The amount of salt, such as ammonium sulfate, that is required to precipitate any given globulin is largely characteristic for the individual globulin. The precipitation limits of different globulins studied in this laboratory cover a wide range. Some of the α -globulins, such as arachin and those of the lima, navy, and mung beans, begin to precipitate from their solutions at concentrations of ammonium sulfate as low as 0.15 of saturation with this salt. Other globulins are known which require for their precipitation as much as 0.7 to 0.8 of saturation. Up to the present time there was not known a lower concentration limit for precipitating proteins with ammonium sulfate than that required for the globulins. Since there is a relationship between protein solubility in aqueous media and the quantity of ammonium sulfate needed for complete precipitation, it seemed probable that glutelins, which are less soluble proteins as a group than globulins and require the use of alkalis to bring them into solution, would need less ammonium sulfate for their precipitation, if they are at all precipitable, than the relatively low limit given above for globulins.

In a recent paper (1) on the isoelectric points of proteins it was pointed out that protein precipitation by ammonium sulfate is a

* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists held in Cleveland, December 28 to 30, 1925.

salting out effect, and that the precipitation is not brought about by a change of pH. Therefore, it was believed that the alkalinity of the glutenin solution would not necessarily interfere with our project. The correctness of this view was supported by the fact that a globulin could be precipitated from either a 0.2 per cent sodium hydroxide solution or a 10 per cent sodium chloride solution by addition of the same amount of ammonium sulfate. These considerations led us to study the possibility of separatingutelins from alkaline solution by precipitation with ammonium sulfate.

It was found that most of the wheat glutenin contained in a 0.2 per cent sodium hydroxide extract of the residue remaining after removal of gliadin from wheat gluten, could be precipitated from the alkaline extract by addition of small amounts of ammonium sulfate, corresponding to 0.018 to 0.02 of saturation. Results of work still in progress have shown thatutelins of other cereals, namely those of rice, corn, and oats, can also be similarly precipitated by correspondingly small quantities of ammonium sulfate. It appears, therefore, that we have here a method applicable not only for characterizingutelins as a class, but also for a more satisfactory separation and preparation of a class of proteins concerning which but relatively little is known. Glutenin, the glutenin of wheat, is the only representative of this class of proteins that has been extensively studied, and only two others, theutelins of rice and maize, can be considered as at all well defined. Glutelins from other sources have been reported but sufficient evidence has not been obtained to justify considering them as even approximately pure preparations.

Glutelins are generally prepared from seed residues that have been exhaustively extracted with neutral salt solutions and alcohol for removing albumins, globulins, and prolamins. Contact with the solvents during extraction frequently has a denaturing effect upon these proteins, rendering them insoluble in neutral solvents. When alkalis are used to extract glutenin from such residues, the denatured proteins will also go into solution. The glutenin preparation obtained by neutralizing the alkaline extract with acid will consequently consist of a mixture of proteins and not of glutenin alone. Such preparations may also contain, as Osborne has pointed out, small quantities of proteins which escaped extraction by neutral solvents, either on account of having

been contained in unruptured cells which were afterward disintegrated by the alkaline solution, or because they were retained in the seed residue in combination with substances such as nucleic acid or tannin, which rendered them insoluble in neutral solvents.

Inasmuch as the glutelins which we have studied thus far have been found to be precipitable from alkaline solutions by quantities of ammonium sulfate that come within rather narrow limits, and are far too small to precipitate globulins under similar conditions, this method makes possible the preparation of glutelins free from significant amounts of these other proteins.

The idea that wheat glutenin as generally prepared is not an individual protein, but consists of more than one glutelin has been previously advanced by several investigators, this view being based chiefly on the physical behavior of glutenin.

Fleurent (2) in 1896 published a method for the quantitative determination of prolamins and glutelins in different cereals. This method consisted of extracting these proteins with a solution of potassium hydroxide in alcohol (0.3 gm. of potassium hydroxide per 100 cc. of 70 per cent alcohol). In the case of wheat, he extracted the gluten, prepared by washing out the starch from the flour with water, with the alcoholic potassium hydroxide solution. By saturating the alkaline extract with carbon dioxide, a precipitate was obtained which he designated as glutenin, a name originally given to wheat glutelin by Osborne. When the filtrate was acidified with sulfuric acid a second precipitate was obtained which he named conglutin. Fleurent states that the yield obtained by the second precipitation does not represent more than 2 to 8 per cent of the gluten and therefore plays only a secondary part in the physical character of gluten. Because no analytical data are given for these glutelin preparations, comparison of them with our preparations cannot be made. Blish and Sandstedt (3) published a quantitative method for the determination of glutenin using the principle introduced by Fleurent of extracting with an alcoholic solution of alkali. They, however, found methyl alcohol preferable to ethyl alcohol, and used hydrochloric acid instead of sulfuric acid for neutralizing the alkali. This procedure would give them both the glutenin and conglutin of Fleurent.

Halton (4) claimed that by fractional precipitation he obtained two glutenins differing in optical rotation. Blish (5) applying

Halton's method to purified glutenin preparations, obtained from flours of different sources, could not confirm the latter's results. In no instance was more than one fraction obtained. Blish offers as an explanation for Halton's results that a partial racemization of the glutenin had occurred before fractionation, which might have produced fractions of the same protein having different isoelectric points. That this was possible was actually shown experimentally.

Woodman (6) determined the racemization curves of glutenins prepared from strong and weak flours. Since the curves differed he concluded that these glutenin preparations were not identical. On the other hand, several investigators (7-11) found little or no difference in the chemical composition of glutenin preparations obtained from different varieties of wheat.

By fractional precipitation with ammonium sulfate, we have obtained from a 0.2 per cent sodium hydroxide extract of the residue remaining after exhaustively extracting wheat gluten with alcohol to remove gliadin, two fractions, the properties and composition of which indicate that they are two different glutelins. The first fraction, which we have designated α -glutelin, separated as a flocculent precipitate when the alkaline extract was made 0.018 to 0.02 saturated with ammonium sulfate. More ammonium sulfate added to the filtrate from this precipitate caused no further precipitation until a quantity had been added sufficient to make the solution 0.16 to 0.18 saturated. This fraction is referred to as the β -glutelin. The α -glutelin was obtained in the greater quantity, the yield being very nearly 7 times that of the β -glutelin obtained.

That we are dealing here with two different glutelins is shown not only by the sharp line of demarcation in their precipitation limits with ammonium sulfate, but also in their nitrogen content and the distribution of nitrogen as determined by the Van Slyke method (Table III). The most striking differences found between these two glutelins are shown in their content of amide nitrogen, arginine, and lysine.

In view of the fact that the yield of the β -glutelin was relatively small it might be urged that this fraction represented the small amount of globulin that is usually present in wheat flour. That this is not the case is indicated by the fact that a third fraction

separated when ammonium sulfate was added to the filtrate from the β -glutelin to 0.3 of saturation. The amount of this fraction corresponded roughly to that of the globulin usually found in flour, and it was precipitated at a concentration of ammonium sulfate well within the limits at which globulins are frequently precipitated. Furthermore, when the gluten had been exhaustively extracted with sodium chloride solution previous to the extraction with alcohol and alkali, thereby removing any globulin that may have been present, no trace of a precipitate was obtained by addition of ammonium sulfate to the filtrate from the β -glutelin. The fact that the β -glutelin contained only 16.1 per cent of nitrogen while the globulin isolated by Osborne and Voorhees (12) contained 18.39 per cent, furthermore renders it improbable that our second fraction consisted of a globulin.

Again, the isoelectric point of the β -glutelin was found to be pH 6.45, identical with that found for the α -glutelin. In the light of the results we obtained when working on the isoelectric points of proteins (1), an isoelectric point of pH 6.5 is much too high for a globulin. It was shown that the isoelectric points of the proteins of any one group fall within rather narrow limits, and that the more soluble proteins, as the albumins, have the lower isoelectric points, while less soluble proteins precipitate with ammonium sulfate at a lower concentration and have higher isoelectric points. The isoelectric points of albumins were found to range in general from pH 4 to pH 5, those of globulins from pH 5 to 5.5, and those of prolamins from pH 6 to 6.5.

The amount of ammonium sulfate required to precipitate the glutelins was found to vary to a certain extent both with the concentration of the alkali solution used and with the concentration of the protein in the extract (Table I). The higher the concentration of alkali, the more ammonium sulfate was required to precipitate the glutelins.

Addition of ammonium sulfate to the alkaline extract containing the glutelins lowers the pH of the solution. However, it still remains on the alkaline side when the precipitation of the glutelins occurs.

It is of interest to note that gliadin can also be precipitated from a 0.2 per cent aqueous sodium hydroxide solution by the addition of even a smaller amount of ammonium sulfate than is required

to precipitate the α -glutelin. At 0.01 of saturation with ammonium sulfate gliadin separates from a 0.2 per cent sodium hydroxide solution as a sticky precipitate

Preparation of the α - and β -Glutelins from Wheat Flour.

2 kilos of wheat flour were kneaded to a stiff dough. After standing for an hour, the starch was washed out with tap water, during which process the gluten was loosely contained in a cheese-cloth bag, in order to avoid loss. The gliadin was then removed by successively extracting the gluten with about 1000 cc. portions of 70 per cent (by volume) alcohol. The ninth extract contained 0.45 gm. of solids which, judging from the small amount of nitrogen

TABLE I

Influence of Concentrations of Protein and Alkali upon Amount of Ammonium Sulfate Required for Precipitation of the α -Glutelin of Wheat.

Concentration of NaOH	Concentration of glutelin		
	0.2 per cent	1.0 per cent	2.0 per cent
	Ammonium sulfate required for precipitation *		
per cent			
0.2	0.04	0.018	0.018
1.2		0.051	
2.2		0.073	

* Expressed in terms of saturation

(14 mg.), was only partly protein material. To hasten the extraction, the gluten was first cut up into small pieces in a meat grinder and allowed to stand in the alcohol for 1 to 2 days with occasional stirring.

After the gliadin had been removed, the residue was stirred for 2 hours with 2 liters of 0.2 per cent sodium hydroxide, then centrifuged, and the supernatant liquid filtered clear through paper pulp. Centrifugation aids materially in the subsequent filtration, as it separates starch and undissolved protein which would clog the filter. To the clear filtrate (1900 cc.) 50 cc. of saturated ammonium sulfate solution were added with constant stirring, whereupon the α -glutelin separated in a flocculent form and settled

quickly. As much as possible of the supernatant liquid (A) was siphoned off and the residue centrifuged, in order to separate more thoroughly the precipitate from the liquid. The precipitate was washed by centrifugation twice with 500 cc. of 0.04 saturated ammonium sulfate solution. It was then dissolved in 0.2 per cent sodium hydroxide solution, and reprecipitated and washed as before. After the last washing with ammonium sulfate the precipitate was washed once with distilled water to which a few drops of dilute hydrochloric acid were added—just enough to bring the liquid in which the precipitate was suspended and well mixed to a pH of 6.4. By this manipulation most of the inorganic salts were removed. The precipitate was finally washed twice with 60 per cent alcohol followed by two washings with 95 per cent alcohol.

After dehydration with absolute alcohol and ether in the usual way, 20 gm. of the glutelin were obtained as a fine, grayish white powder.

The β -glutelin was obtained from the liquid (A) which had been siphoned off from the precipitate representing the α -glutelin. The liquid was filtered through a mat of paper pulp and enough saturated ammonium sulfate solution was added to the clear filtrate to make it 0.18 saturated. The resulting precipitate was then purified by reprecipitation and treated in the same manner as the α -glutelin, with the exception that 0.2 saturated ammonium sulfate solution was used for the first two washings. A yield of 1.35 gm. of air-dried material was obtained.

A somewhat better yield of the β -glutelin was obtained by a modified method wherein the extraction with 70 per cent alcohol to remove the gliadin was omitted. The gluten prepared from 1 kilo of flour was dispersed in 3 liters of 0.2 per cent sodium hydroxide and 60 to 80 cc. of saturated ammonium sulfate solution added. This precipitated both the gliadin and the α -glutelin. From the supernatant liquid the β -glutelin was separated and prepared as described above. A yield of 1.54 gm. was obtained.

The composition and distribution of the nitrogen of the two glutelins are shown in Tables II and III. The figures given represent the average of duplicate determinations. Inasmuch as our preparations represent two different glutelins, these analytical figures obviously differ from those found in the literature for

TABLE II
Composition of Wheat Glutelins

	α -Glutelin	β -Glutelin	
		Sample 1	Sample 2 *
Nitrogen (moisture- and ash-free basis)	17 14	16 10	16 03
Sulfur (" " " ")	1 59		
Ash (moisture-free basis)	0 142	0 684	0 530

* This preparation was obtained from gluten which had not been previously extracted with alcohol to remove gliadin.

TABLE III
*Distribution of Nitrogen in Wheat Glutelins as Determined by the Van Slyke Method **

	α -Glu- telin	β -Glu- telin	Amino acids expressed in percentages of the ash- and moisture-free proteins		
	<i>per cent</i>	<i>per cent</i>		α -Glu- telin <i>per cent</i>	β -Glu- telin <i>per cent</i>
Amide N	17 8	11 06	Cystine	3 25	7 49
Humin "	1 05	1 32	Arginine	5 83	3 05
Cystine N	1 76	5 43	Histidine	3 48	3 67
Arginine N	10 95	6 10	Lysine	2 76	5 75
Histidine "	5 50	6 17			
Lysine N	3 09	6 85			
Amino " of filtrate	45 4	49 13			
Non-amino N of filtrate	13 0	14 9			

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

glutenin, a term used to designate the total glutelin fraction of wheat.

The isoelectric points of the proteins, which were found to be identical (pH 6.45), were determined according to the method described in a previous publication from this laboratory (1).

SUMMARY.

It has been found thatutelins can be precipitated from a 0.2 per cent sodium hydroxide solution by addition of small amounts of ammonium sulfate. By this method two glutelin frac-

tions have been isolated from wheat gluten. The α -glutelin separates as a flocculent precipitate by making the alkaline solution 0.018 to 0.02 saturated with ammonium sulfate. The β -glutelin does not begin to precipitate at a concentration of ammonium sulfate less than 0.16 of saturation, and requires about 0.18 of saturation for complete precipitation.

The two glutelins differ also in their chemical composition. The α -glutelin contains 17.14 per cent nitrogen, and the β -glutelin 16.06 per cent. Analysis of the glutelins by the Van Slyke method gave the following results expressed as percentages of the total nitrogen: α -glutelin: amide N 17.8, cystine N 1.76, arginine N 10.95, histidine N 5.50, lysine N 3.09; β -glutelin: amide N 11.06, cystine N 5.43, arginine N 6.1, histidine N 6.17, lysine N 6.85.

Both glutelins have the same isoelectric point, namely pH 6.45.

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THE ACTION OF ALKALI AND HYDROGEN PEROXIDE ON GLYOXALS.

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Although it is known that glyoxals are converted by alkali into the corresponding hydroxy acids (1-4) and on oxidation (1, 3, 5) yield formic and other acids, the extent of these conversions has apparently not been determined. The experiments cited below show that the conversion under the two conditions, (1) action of alkali (3) and (2) action of alkali and H_2O_2 , is substantially quantitative. Glyoxal yields with alkali just one-half as much acid (glycollic) as when oxidized by peroxide to formic acid. Methylglyoxal yields with alkali the theoretical amount of lactic acid and with peroxide twice as much acid, the acids consisting of equivalent amounts of acetic and formic acids. Glyoxal carboxylic acid gives with alkali and peroxide just twice as much acid (probably oxalic and formic acids) as is formed by alkali alone. Triosone, hydroxymethylglyoxal, likewise yields twice as much acid (probably glycollic and formic acids) with alkaline peroxide as with alkali alone.

The relation between the reaction rates in alkali and in alkaline hydrogen peroxide is of considerable interest. The rate of oxidation of methylglyoxal and glyoxal (Shaffer and Friedemann (3)) by peroxide is much more rapid and occurs at lower pH than the conversion to hydroxy acid. Both glyoxals require a high alkalinity for very rapid conversion to hydroxy acid; but if H_2O_2 is present oxidation occurs rapidly at pH 7 or less.¹ If one adds

¹ The oxidation in fact is so rapid that lactic acid determinations of solutions after peroxide oxidation in $\text{N}/2$ KOH showed no measurable increase of lactic acid above that already present in the methylglyoxal solution itself. It is evident from this that lactic acid is not formed in the

dilute NaOH to a solution of methylglyoxal until alkaline to phenolphthalein, the pink color persists for some time unless shaken with air, when it fades more rapidly due to formation of acid by air oxidation. This shows that hydroxy acid formation is quite slow at pH 8 to 9, although it proceeds rapidly at pH 12 or above. But if neutral H_2O_2 is added to the above solution the pink color disappears instantly and the addition of methyl red or methyl orange shows that oxidation goes on rapidly until a pH of about 4 is reached, when it slows up and soon stops.

By continual addition of alkali in the presence of peroxide the glyoxal may be oxidized completely and the acid titrated as formed, with phenolphthalein as the indicator. The solution does not become pink, even with very rapid addition of N/10 or other alkali, until 95 to 98 per cent is oxidized; standing a few minutes with a slight excess of alkali completes the oxidation, after which the excess alkali may be titrated with acid and the glyoxal thus determined quantitatively.

Glyoxal Carboxylic Acid—A solution of this substance was prepared as directed by Fenton (2) by oxidizing dihydroxymaleic acid with HgCl_2 . The solution, freed from mercury, contained much HCl and perhaps glyoxal (2).

10 cc of the solution were first neutralized to phenolphthalein and then a measured excess of N/10 NaOH was added. The solution was heated about 5 minutes on the electric hot plate and then cooled. It was titrated by N/10 HCl until colorless to phenolphthalein.

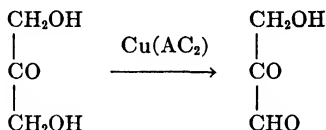
Another 10 cc portion was neutralized to phenolphthalein. Neutral H_2O_2 was added and an excess of N/10 NaOH was run in. After standing 10 minutes at room temperature it was titrated by N/10 HCl until colorless.

Gain of acidity due to NaOH	8 4 cc. N/10
" " " " H_2O_2 + NaOH	16 9 " "

Triosone (Hydroxypyruvic Aldehyde or Hydroxymethylglyoxal).—The preparation of this substance had not been described when this work was undertaken. Since the glyoxals are relatively stable

presence of hydrogen peroxide and is therefore only an *asphyxial product*, as it is also in alkaline sugar solutions and perhaps in muscle and other tissues

toward oxidizing agents in acid solutions (perhaps due to polymerization) it was thought that dihydroxyacetone would be oxidized to triosone in a manner similar to the oxidation of acetol by copper acetate described by Denis (1). It has since been shown by Evans and Waring (6) that triosone is the sole product of this oxidation.



Dihydroxyacetone was oxidized by an excess of finely powdered copper acetate suspended in water. The flask was vigorously shaken at intervals during the 1st day, thereafter it was shaken at about 8 hour intervals. The separation of Cu_2O began almost immediately but proceeded very slowly. The oxidation appeared complete after 7 days at room temperature. The precipitate was filtered off and the solution was freed from copper by H_2S , and from H_2S by aeration.

10 cc. aliquots of the solution were treated with $\text{N}/10$ NaOH and $\text{H}_2\text{O}_2 + \text{N}/10$ NaOH as described above in the experiments with glyoxal carboxylic acid.

Gain of acidity due to NaOH	9.4 cc. $\text{N}/10$
" " " " $\text{H}_2\text{O}_2 + \text{NaOH}$	19.3 " "

Methylglyoxal.—Methylglyoxal was prepared from dihydroxyacetone according to the method of Fischer and Taube (7). The product, a clear pale green liquid, was immediately diluted with water and made up to about $\text{M}/2$ strength. The solution was acid, due to the presence of lactic acid and a small amount of phosphoric acid.

In Table I the yield of total acid formed in alkali is compared with the yield of lactic acid, the latter being determined by the acid KMnO_4 method (8). The results are substantially identical. Results are also given for total acid, volatile acid, formic acid, and acetic acid yielded by peroxide oxidation.² The hydrogen peroxide consumption is also given. It is evident that (1) the total acid on oxidation is twice the amount formed by alkali alone,

² The analytical procedure was the same as that described by Shaffer and Friedemann (3).

TABLE I

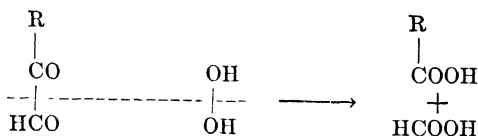
Products Obtained by the Action of 0.5 N KOH and 0.5 N KOH + H₂O₂ on Methylglyoxal

The results represent cc of N acid or millimols from 100 cc. of methylglyoxal solution.

	Solution 1		Solution 2	
	KOH	KOH + H ₂ O ₂	KOH	KOH + H ₂ O ₂
Total acidity	41.0	88.8 90.0	45.0	89.5 93.1
Volatile acids		84.7		91.3
Formic acid		42.3		45.65
Acetic " (by difference)		42.4		45.65
H ₂ O ₂ consumed				48.2
Lactic acid	41.6		45.5	
Ether-soluble acid			46.2	
Total acidity with H ₂ O ₂ + slight excess N/10 NaOH				90.90
				90.84

that (2) almost all of the acid is volatile, that (3) equivalent amounts of formic and acetic acids are formed, and that (4) 1 molecule of hydrogen peroxide is required for the oxidation.

The glyoxals (and perhaps also ozones) in general seem to be oxidized by hydrogen peroxide as follows with the formation of 2 molecules of acid:



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THE DETERMINATION OF LACTIC ACID.*

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As the result of some experience in the determination of lactic acid in blood, muscle, and solutions of sugar derivatives, we have introduced various modifications in the process by which it gains considerably in simplicity, speed, convenience, and reliability. The basis of the method is the oxidation of the lactic acid in boiling sulfuric acid solution to acetaldehyde by permanganate (1), aeration of the aldehyde into large excess of sodium bisulfite solution, and titration of the combined bisulfite as described by Clausen (2). The chief modifications which we introduce are: (1) the form of apparatus used for the oxidation and for the collection of the aldehyde, (2) the addition of manganous sulfate to catalyze the oxidation by permanganate, and (3) more accurately defining the conditions for the titration of the sulfurous acid bound by the aldehyde. We have tested separately the several stages of the method and have checked it on known amounts of lactic acid in pure solution and after addition to blood. A number of other substances have been tested as to the extent of their interference in the determination of lactic acid.

By the procedure as herein described the yield of aldehyde is consistently 96 to 98 per cent of the theoretical; and routine results are, in our hands, not subject to the variability and uncertainty commonly experienced with lactic acid determinations by older methods. The process is also much shorter in time and

* This paper includes data to be presented by one of the authors (M C) in a dissertation for submission to Washington University

is to a considerable extent free from certain bothersome details¹ which heretofore have imposed serious handicaps in the determination of this substance. The oxidation, collection of aldehyde, and titration may be completed in from 15 to 20 minutes, or about one-third to one-fifth the time required by older methods.² With four sets of apparatus two persons have made more than 60 determinations in 8 hours. The technique is readily adapted to the determination of widely different quantities, and is to this extent rather generally applicable. Besides being a useful analytical method, the reactions involved present several interesting theoretical points which are considered.

Apparatus.

The apparatus, which is simple and inexpensive (Fig. 1), consists essentially of a boiling flask fitted to a reflux condenser through which an air current carries the very volatile aldehyde into an absorbing tube or tower containing bisulfite. Instead of the bead tower shown in the figure two large test-tubes in tandem may be used for the absorption. The whole apparatus may readily be constructed from ordinary flasks, test-tubes, and glass tubing. Besides being a convenient form in which to conduct the reaction, the apparatus accomplishes two main purposes, (1) the rapid removal of the aldehyde by the air current without distilling liquid into the receiver, thereby keeping the volume small for the subsequent titration, and (2) a fairly efficient separation of acetaldehyde from other less volatile sulfite-binding products which may result from the oxidation of substances other than lactic acid. The number of interfering substances is thus reduced and the method made more nearly specific for lactic acid (or sub-

¹ See for example the precautions advised by Embden (*Z. physiol. Chem.*, 1925, cxliii, 297) who prescribes that the oxidation of 3 mg of lactic acid should require 1½ hours; (also Hirsch-Kauffmann, *Z. physiol. Chem.*, 1924, cxl, 30, 45).

² With small amounts of lactic acid, as in blood analysis, it is quite possible to obtain equally high yields without added MnSO_4 , provided the rate of KMnO_4 addition be very slow and well regulated, as shown for example by the work of Hirsch-Kauffmann. In such circumstances the effect of MnSO_4 merely permits more rapid oxidation without the sacrifice of accuracy which otherwise occurs.

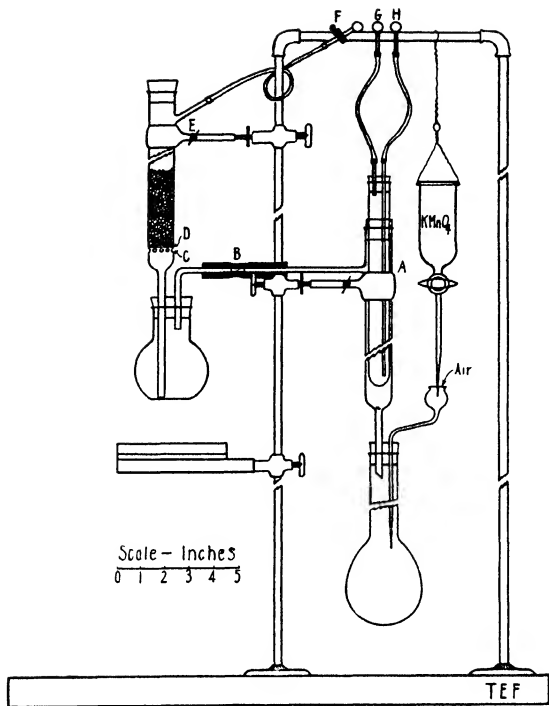


FIG 1 A is a reflux condenser of the Hopkins type, connected with a 300 cc Kjeldahl flask by a stopper which carries also a dropping funnel through which enter the air current and KMnO_4 solution. The flask is supported from the condenser and needs no separate clamp. For most efficient operation the space between the inside condenser tube and the inner wall of the jacket should be only 1 to 3 mm and the rate of water flow sufficient to keep the air in A and B cold. The condenser may be made of large Pyrex test-tubes. C, D, E is a tower of glass beads, connected to a 150 cc wide mouth extraction flask which contains the bisulfite solution, the whole being connected to the condenser by a rubber tubing at B. The tower is constructed from a large Pyrex test-tube by sealing on tubes, and making indentations at C to hold a perforated glass or porcelain plate at D, which supports an 8 cm column of beads. A tube from a water pump connected at E provides the air current. The entire apparatus is mounted permanently by burette clamps A and E. The clamp at A (Arthur H. Thomas Company catalogue No. 3220) is kept loose at its base so as to permit the condenser being swung sidewise when the Kjeldahl flask is put in place. At the end of the oxidation the aeration is stopped by removing the stopper from the top of the tower, the extraction flask is detached and lowered to the shelf below it, and the tower and beads washed with five to seven 5 cc portions of water. The flask is then removed for the titration. If many determinations are to be run it is convenient to set up a series of these units; we use six, which may be operated by one person.

stances which yield under the conditions very volatile bisulfite-binding products).

Action of Manganese Sulfate.

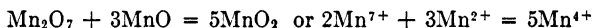
The presence of an excess of manganous salt at the start of the oxidation of lactic acid has a remarkable effect in greatly increasing the rate of oxidation by permanganate, and in increasing the yield of aldehyde. The effect of increasing the rate of oxidation is doubtless the same as that often observed in the titration of oxalic acid, and is of interest apart from its use in the lactic acid method. In both cases the oxidation, in the absence of manganous salt, proceeds at first very slowly and rapidly increases after some of the added permanganate is reduced, but if manganous salt be added before the permanganate the latter reacts almost instantly with the result that the lactic acid is virtually *titrated* (as with oxalic acid), an excess of permanganate consequently does not accumulate until toward the end, and the danger of further oxidation of aldehyde by excess permanganate is thus minimized. Rapid removal of the aldehyde from the boiling liquid by the air current is an additional protection.

The action of manganous salt thus appears to be ideally suited to the purpose at hand. Besides increasing markedly the rate of the oxidation, it tempers or restrains the reaction so as to avoid secondary oxidations which result in products other than acetaldehyde. This latter effect considerably reduces the importance of a very slow addition of permanganate which is otherwise so essential to obtain correct results.

The effect first named, that of increasing the rate of oxidation, is very evident in the actual process of determination if one compares the rate of permanganate consumption by lactic acid in the presence and absence of added manganous sulfate; and is more simply demonstrated by noting the rate at which the color fades when a few drops of dilute KMnO_4 are added to acidified solutions of lactic (or oxalic) acid, with and without added MnSO_4 . At room temperature the pink color of the solution containing manganous salt fades at once with oxalic acid, or after a few minutes with lactic acid, while without the MnSO_4 the pink persists much longer.

The second effect, that of increasing the yield of acetaldehyde is shown by analytical data. Under comparable conditions 5 to 20 per cent more aldehyde may be obtained with MnSO_4 than without. As an illustration of this effect the following results may be cited. From the oxidation of 109 mg. of lactic acid in $\text{N H}_2\text{SO}_4$ by dropping into the boiling liquid 0.1 N KMnO_4 and simple distillation of the acetaldehyde into bisulfite solution, the average yield without added MnSO_4 was only 67.5 per cent, while with the addition of 5 to 50 cc. of 5 per cent MnSO_4 the yields rose to 85 per cent of the theoretical value. The low results even with protective action of manganous salt also illustrate the fact that simple distillation is much less effective than aeration plus distillation, which with the aid of manganous salts yields consistently 96 to 98 per cent.²

The explanation of the action of manganous salt seems fairly clear. It is due to the reaction between permanganate and manganous salts which results in the formation of manganite or MnO_2 which is the effective oxidizing agent of lactic acid.

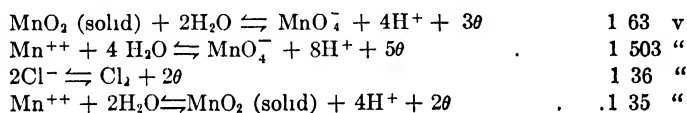


In this reaction by an exchange of electrons, the permanganate (Mn^{7+}) is transformed to a lower intensity level (Mn^{4+}), which is still high enough to oxidize the lactic acid,³ but not so intense as to oxidize rapidly the acetaldehyde. The reaction between Mn^{2+} and Mn^{7+} takes place with the separation of hydrated manganite or MnO_2 if no oxidizable substance is present, but in the presence of lactic acid or other oxidizable substance the solution remains clear due to reduction of the Mn^{4+} to soluble salts of Mn^{2+} . In either case the Mn^{7+} is removed and oxidations which might result from its action are thus prevented. If one adds a drop of dilute KMnO_4 to a solution of acetaldehyde in $\text{N H}_2\text{SO}_4$, and the same amount also to another portion of aldehyde solution containing MnSO_4 , the color fades more rapidly in the solution *without* MnSO_4 , while a precipitate forms in the other which persists. This evidently means that Mn^{7+} (KMnO_4) oxidizes acetaldehyde while Mn^{4+} (MnO_2) does not do so, or but slowly.

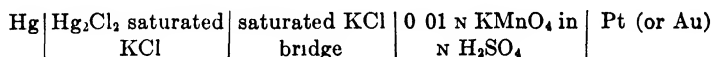
³ As observed long ago by Boas (1) MnO_2 oxidizes lactic acid

The MnSO_4 therefore protects the aldehyde from oxidation, by removing the Mn^{7+} (MnO_4^-).⁴

Why the MnO_4^- alone oxidizes the lactic acid (or oxalic acid or other substance) more *slowly* than does the lower MnO_2 is not evident, though presumably it is due to the electronic condition of the molecule containing Mn^{7+} (MnO_4^-).⁵ That the general explanation advanced above is correct seems to be indicated by a consideration of the electric potentials of the systems. The following values are given (Kolthoff (5)) as the normal potentials, referred to the normal hydrogen electrode, for the electrode reactions stated.



A few observations of our own of the chain



gave values at 25°C varying in different trials from 1.18 to 1.26 v, sometimes the Pt and sometimes the Au being the higher. Converting these values with reference to the normal hydrogen electrode we have + 1.426 to 1.507 v. The addition of approximately 0.05 M MnSO_4 caused a drop of potential to 1.11 (or

⁴ A somewhat related phenomenon is probably that long ago noted in the titration of ferrous iron by permanganate in the presence of chlorides (for discussion see Treadwell, F. P., Quantitative analysis, New York, 2nd edition, 1910). In the presence of chlorides (and many other substances also) more KMnO_4 is required than corresponds to the oxidation of ferrous to ferric salt, due to oxidation of Cl^- to Cl_2 , "induced" by the peroxide-like form of iron which is the primary product (3, 4). This induced oxidation of Cl^- is however prevented if manganous salt be added before the permanganate. Here one may suppose with Manchot (3) and others that the Mn^{++} is oxidized by the iron peroxide (as well as by MnO_4^-) thus removing the latter and thereby preventing its action on Cl^- .

⁵ If, as given by Lewis, the electron orbits of the Mn^{7+} atom, contain 2, 8, and 8 electrons respectively, this condition should be a more stable arrangement (being that of A, K^+ , Ca^{++} , etc.) than that of Mn^{4+} , with orbits of 2, 8, and 11 electrons (Lewis, G. N., Valence and the structure of atoms and molecules, New York, 1923).

+ 1.357 to N H⁺ electrode) both electrodes coming into agreement within a few millivolts. These figures therefore demonstrate the drop in oxidation intensity which results from the addition of Mn⁺⁺ salt.

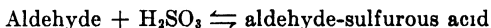
Although the oxidation intensity or potentials required to oxidize lactic acid or acetaldehyde are unknown, it may perhaps be inferred from the observations mentioned earlier that lactic acid begins to be oxidized rapidly under our experimental conditions, below + 1.35 v, while to oxidize acetaldehyde at a comparable rate under the same conditions requires a higher potential. It seems probable that the potential for the oxidation of acetaldehyde (in H₂SO₄ solution) like that for the oxidation of Cl⁻ to Cl₂ lies somewhere between the potentials characteristic of Mn⁷⁺ and Mn⁴⁺. According to the value cited above the potential of Cl⁻ ⇌ Cl₂ is 1.36 v, which is exceeded by Mn⁷⁺ but not by Mn⁴⁺. Quite in accord with expectations based on their relative potentials, although the addition of KMnO₄ to boiling N H₂SO₄ containing HCl rapidly liberates Cl₂, no chlorine is formed if MnSO₄ be added before the KMnO₄. After making this experiment we find that the same facts were noted by Kessler in 1863.

From the above observations we may arrange the apparent oxidizing intensity of these systems in the following order.

Increasing oxidation intensity		Approximate E _A in N H ₂ SO ₄
	KMnO ₄	+ 1 50 v
	Required to oxidize acetaldehyde, less than	+ 1 50 "
		(about + 1 35 (?)).
	Cl ⁻ ⇌ Cl ₂	+ 1 36 v.
	MnO ₂ + Mn ⁺⁺	+ 1 35 "
	Required to oxidize lactic acid, less than	+ 1 35 "

Titration of Sulfite Bound in Acetaldehyde-Sulfite.

The equilibrium in the reversible reaction between acetaldehyde and sulfurous acid



depends upon the reaction of the solution as well as upon the concentration of the components.

In acid solutions nearly all of the aldehyde (or sulfurous acid)

is combined as aldehyde-sulfurous acid in which form both components are stable. The combined sulfurous acid is not oxidized by iodine. Furthermore the *rate* of dissociation of the aldehyde-sulfurous acid is, in acid solution, so slow that any excess of free sulfurous acid may be removed without appreciably disturbing the equilibrium during the short period occupied by the titration. This permits the determination of aldehyde by its addition to a known amount of alkali bisulfite and titration of the excess uncombined sulfurous acid by iodine, as in the Ripper (6) method. At the end-point of this titration there is left in the solution the combined aldehyde-sulfurous acid, HBSO_4 (from the oxidation of the excess HBSO_3) and HI (from the reduction of I_2).

Clausen (2) noted that the addition at this point of weak alkali such as NaHCO_3 causes the disappearance of the blue starch-iodide color and permits the titration of the combined sulfite by the further addition of I_2 , due to the gradual dissociation of the aldehyde-sulfurous acid, which although too slow for titration in acid solution takes place with greater speed in less acid or alkaline solution. If sufficient alkali be present to neutralize the HI and NaHSO_4 formed, the dissociation is relatively rapid and continues to completion if sufficient I_2 be added and sufficient time allowed. Under suitable conditions the Ripper and Clausen titrations may therefore be performed on the same solution, thus giving two determinations, one by difference and one direct, on a single sample.

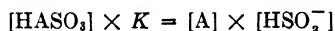
The Clausen plan of titrating the combined sulfite is preferable to the Ripper method for several reasons. (1) It is a direct titration rather than by difference; (2) it allows the use of a large excess of bisulfite to insure complete absorption of the aldehyde and its rapid conversion to the compound, (3) it avoids the errors caused by air oxidation of the uncombined sulfite and loss by volatilization of SO_2 , both of which may cause serious errors in the Ripper titration; (4) it permits the use of aeration to remove the aldehyde from the oxidation mixture, which is impractical with the Ripper titration because of (3) and (2). As already noted, aeration is very desirable because it aids in rapidly sweeping the aldehyde out of contact with permanganate; and when used with a reflux condenser, as in our type of apparatus, the aeration allows a separation of acetaldehyde from other less volatile bisulfite-binding

or iodine-reacting substances which sometimes arise from the oxidation of substances other than lactic acid.

In order to secure these advantages it must, however, be established: (1) That at the first end-point (the start of titrating the bound sulfite) substantially all of the aldehyde is bound. For if, as might be expected, the combination were incomplete or if any considerable dissociation of the aldehyde-sulfite were to occur in acid solution during removal of excess bisulfite in the first titration, that portion would be lost in the subsequent titration. (2) That the dissociation of the combined sulfite is complete during the second titration; and (3) that the liberated sulfite may be determined correctly by titration with iodine in the presence of the added alkali, without the occurrence of side reactions to affect the results. Under suitable conditions these points cause no error, and the titration can be made quite accurate. We shall consider the above points in order.

Proportion of Aldehyde Bound by Bisulfite.

In acid solution the combination of acetaldehyde and sulfurous acid or alkali bisulfite in excess, although by no means instantaneous, is substantially complete if sufficient time be allowed to attain equilibrium. In the presence of large excess bisulfite equilibrium is reached in 10 minutes or less at room temperature, a period well within the time taken for the oxidation of lactic acid and aeration of the aldehyde. The reverse reaction, the dissociation of the compound, is so slow in acid solution that the equilibrium is not perceptibly disturbed during the removal of excess bisulfite by iodine titration. Kerp and Kerp and Baur (7) found values of about 2×10^{-6} for the equilibrium constant (K) in the equation for this reaction



in solutions of M to $M/30$ sodium acetaldehyde-bisulfite at 25°C . The values of K were not very different in the presence of an equivalent excess of aldehyde or $M/30$ HCl or both.

The amounts of lactic acid frequently present in solutions being analyzed are from about 20 to 0.2 mg ⁶ or from 0.2 to 0.002 mM and

⁶ The amounts which may be analyzed are by no means limited to these quantities; see Table III.

at the time of titration the corresponding amounts of aldehyde are contained in about 50 cc. of solution which is therefore 4×10^{-3} to 4×10^{-5} molar as to total aldehyde. With only equivalent concentrations of bisulfite the calculated percentage of uncombined aldehyde at these concentrations would be 2 per cent and 20 per cent. Solutions of pure crystalline acetaldehyde bisulfite show this order of dissociation. But in the presence of 5- and 500-fold excess of bisulfite respectively (10 cc. of 0.1 M solution) these calculated fractions are reduced to 0.01 per cent. With 1 equivalent of aldehyde in excess Kerp found only 0.04 per cent of the sulfite uncombined at 25°C. We have obtained similar results. With 100 cc. of a fresh solution (approximately 0.005 M) of pure crystalline acetaldehyde bisulfite to which were added about 2 equivalents of *excess* acetaldehyde, after standing at room temperature a few minutes, the free sulfite was titrated with 0.01 N I_2 . 2 drops gave a distinct blue end-point, which persisted for several minutes. Making allowance for a blank, the free sulfite amounted to not more than 0.06 per cent of the total present (calculated value about 0.02 per cent). An excess of bisulfite has the same effect in decreasing the uncombined aldehyde as shown by the data in Table II. It is evident therefore that combination is substantially complete.

In acid solution the rate of dissociation after oxidation of the excess bisulfite is so slow that it may safely be ignored. Experiments to test this point showed that in about 0.002 M solution after titration (and oxidation) of the excess free sulfite, the aldehyde-bisulfite dissociated (in the presence of an excess of iodine, which oxidized the sulfite as fast as liberated) only at the rate of about 1 per cent in 30 minutes at 25°. From this we infer that not more than 0.1 per cent of the total will dissociate in the 3 or 4 minutes required for the first titration and adjustment of the first end-point. This conclusion is confirmed by the data cited later which show almost theoretical results for the titration, under optimum conditions, of the bound sulfite in solutions of pure acetaldehyde-bisulfite. (Table II.)

Dissociation of Bound Bisulfite by Alkali.

The next step in the Clausen titration is the addition of alkali to liberate the combined sulfite. Clausen states ((2) p. 265) merely

that, "Sufficient saturated sodium bicarbonate solution is then added to discharge the blue color," and cautions against too high alkalinity from too much bicarbonate, or from carbonate in the bicarbonate, which leads to "secondary reactions, such as the formation of iodoform." These directions need amplification, especially if more than very small amounts of aldehyde are present. The rate at which the compound dissociates depends upon the pH of the solution, which is constantly becoming more

TABLE I.

Effect of Alkali upon Titration of Sodium Bisulfite Solutions by Iodine.

The solutions were titrated in a total volume of about 50 cc.

Alkali added	Titration Cc of I_2 0.1 N	Error
		<i>per cent</i>
A $NaHSO_3$ solution		
No added alkali.	22 20	
Excess solid $NaHCO_3$	21 35	-3 8
Slight excess Na_2CO_3	20 10	-5 0
$NaOH$ to neutralize acid formed. .	20 50	-3 1
B $NaHSO_3$ solution		
No added alkali	40 10	
+glycerol, 0.5 per cent	40 10	0
$NaHCO_3$ + glycerol.	39 30	-2 0
$NaCO_3$ + "	39 85	-0 6
C $NaHSO_3$ solution.		
No added alkali	43 85	.
Added excess acetaldehyde		
Aldehyde + $NaHCO_3$	43 85	0
" + Na_2HPO_4	43 90	+0 1

acid during the titration due to the HI and $HBSO_4$ formed. To secure *complete* dissociation (within short time) a higher pH is required than to cause partial dissociation, due doubtless to the influence of the increasing free aldehyde concentration.

If an insufficient amount of $NaHCO_3$ or other alkali be added the color may at first be discharged, yet the rate of liberation of sulfite and the consequent rate at which the titration with iodine may be carried on may be tediously slow, and may even reach an apparent end-point long before dissociation is complete. If on

the other hand too much alkali be added, with consequent too high pH during the titration, the sulfite is liberated rapidly but *low* rather than high results are obtained. This error is due mainly to air oxidation of sulfite, which occurs more rapidly in alkaline than in acid solution, and is much more serious than the danger of high results from the formation of iodoform noted by Clausen; although loss of iodine by conversion to hypoiodite may become important if the I_2 be added very rapidly in the presence of too great excess of a stronger alkali, *i.e.* too high pH. Obviously the optimum amount and strength of alkali to be added must be such as to avoid the two extremes, to permit complete liberation of the sulfite within a convenient period of titration without producing a dangerous alkalinity.

Without aldehyde the titration of sulfite by I_2 in presence of excess $NaHCO_3$, Na_2CO_3 , or other alkali yields low results (see Table I); and that these low results are due to air oxidation of sulfite we have shown by determination of sulfite before and after aeration of the alkaline sulfite solutions. The presence of the aldehyde markedly protects the sulfite from air oxidations, by forming the stable compound. But this protective action of the aldehyde is in part lost when as a result of too high alkalinity the dissociation takes place much faster than the I_2 is added and the free sulfite is thus exposed to air. The ideal would therefore be to add gradually throughout the titration parallel with the I_2 an amount of alkali just sufficient to neutralize the HI and $HBSO_4$ formed in the oxidation by I_2 and thus to liberate the sulfite at about the rate at which it is titrated with I_2 .⁷

Bearing in mind the limitations stated above and the fact that $1\frac{1}{2}$ equivalents of acid ($1\text{ HI} + 0.5\text{ HBSO}_4$) are formed for each

⁷ A curious fact for which we are not able to offer an explanation is the following. After titrating the excess bisulfite of an aldehyde-sulfite solution, a drop of 2 or 0.1 N NaOH instantly discharges the blue color of the starch end-point, in spite of the fact that the solution is still strongly acid from the HI and $HBSO_4$ formed in the first titration. Further addition of I_2 restores the blue, to be discharged again by a few drops of dilute alkali. The addition of 10 or 20 cc. or more of 0.1 N HCl to the solution, thus further increasing its acidity, does not prevent this immediate disappearance of the blue on subsequent addition of a drop or 2 of 0.1 N alkali. The effect can therefore hardly be due simply to pH change. (In the absence of aldehyde-sulfite the alkali, of course, has no such effect on the blue iodide of starch.)

normal equivalent of I_2 used in the titration, one may add the corresponding amounts of alkali in various forms, provided that the alkalinity in the early stages of the titration is sufficient only to allow dissociation at a rate approximately that of the rate of titration, thereby avoiding the presence of excess free sulfite to be oxidized by air, and providing that toward the end the residual alkalinity is high enough to allow complete dissociation.

Analysis of Acetaldehyde Sodium Bisulfite.

The criteria for choice of alkali are obviously correct results and convenience. By the use of pure aldehyde-sodium bisulfite we have a means of accurately testing the titration. Data are given below for the analysis of a very pure sample of this compound for the preparation of which we are indebted to Mr. Earle Adler.⁸

The figures given for sodium and sulfur content agree well with the calculated values for the compound containing $\frac{1}{2}$ mol of water of crystallization, and indicate the purity of the preparation. Under optimum conditions the results of titration of the combined sulfite (and thus the aldehyde) with standard iodine by the Clausen principle also agree very well, as shown by the results of

⁸ Because of the purity of the product as well as because of failure in several earlier attempts we record the successful procedure. A fresh concentrated solution of sodium bisulfite is made by passing SO_2 , generated by dropping 125 cc. of 1 N H_2SO_4 into a solution of 150 gm. of Na_2SO_3 in 250 cc. of water, into a solution of 60 gm. of pure Na_2CO_3 in 150 cc. of water. The solution must contain an excess of SO_2 , indicated by a greenish color. The bisulfite solution in a 2 liter flask is well cooled in ice-salt mixture; to it is added, very slowly and with shaking, 45 gm. of very cold acetaldehyde (Mallinckrodt's preparation containing 9 per cent alcohol was used). After standing several hours in the freezing mixture about 1500 cc. of chilled 95 per cent alcohol are added in small portions over a period of an hour. The mixture is kept thoroughly chilled and is stirred or shaken at intervals. Soon after the last portions of alcohol are added the mixture sets to a viscous mass of crystals, which is then filtered rapidly by suction on a previously chilled Buchner funnel and sucked fairly free from mother liquid. The crystalline material is then dissolved in the smallest possible amount of water (75 to 100 cc.), the solution is well cooled in freezing mixture, and about a liter of alcohol is added in portions as before. After crystallization the mixture is filtered on a cold funnel, and again recrystallized. The product is finally dried in air at room temperature to constant weight. Yield, after two recrystallizations about 135 gm. The material is free from sulfates and as shown by the data cited has the composition $NaSO_3 \cdot C_2H_5O \cdot \frac{1}{2}H_2O$.

such titrations, calculated as equivalent per cent of aldehyde in the substance, given under aldehyde. The accuracy of the Clausen titration is thus demonstrated.

The preparation was substantially free from sulfate. Determinations of sodium, sulfur, and aldehyde (combined sulfite determined by iodine titration) gave the following results.

	Sodium	Sulfur	Aldehyde
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Theory for $\text{NaHSO}_3 \cdot \text{C}_2\text{H}_4\text{O} \cdot \frac{1}{2} \text{H}_2\text{O}$	14 64	20 40	28 02
Found	14 60	20 52 20 48 20 70	27 90 28 00 28 10
Average found	14 60	20 57	28 00
Per cent of theory	99 73	100 7	99 93

In Table II is given a summary of a number of titrations of bound sulfite (or aldehyde) in solutions of widely different concentrations, and with different alkalies to liberate the sulfite. All of the titrations on solutions of the pure compound were made under conditions simulating those in the lactic acid determination. Weighed amounts of the substance were dissolved to known volumes and aliquots of the solutions pipetted into sodium bisulfite solutions of such concentration as to make about 0.02 to 0.05 M excess bisulfite after dilution. The solutions titrated thus contained large excess of bisulfite which prevented dissociation of the aldehyde compound and which also liberated acid in the first titration (of the excess free sulfite) thereby regulating the reaction, as noted below, when the alkali was subsequently added. Without attention to these points the results for bound sulfite are low for the reasons already stated. The volume of the solutions titrated was 40 to 80 cc. As indicated by the data in Table II, the results vary by not more than about ± 1 per cent or less at widely different concentrations, except at great dilution when the results are 4 to 8 per cent too high.

Choice of Alkali.

Of the alkalies tried, sodium bicarbonate, used by Clausen, yields correct results and is very convenient. With it the danger

of too great alkalinity is slight and with liberal amounts the titrations may be quite rapid unless the temperature of the solutions is too low. When, as occurs under conditions here described for the lactic acid determination, the large excess of bisulfite gives rise in the first titration to HI and HBSO_4 , this acid prevents dangerous alkalinity from the subsequent addition of even large excess of solid bicarbonate. When the first titration is 5 to 10 cc. of 0.1 N I_2 sufficient of the liberated CO_2 remains dis-

TABLE II

Results of Titration of Solutions of Pure Acetaldehyde-Bisulfite (with Excess of Bisulfite) after Addition of Various Alkalies

20 or 25 cc of concentrations stated were titrated in total volume of about 50 cc

Alkali used to liberate bound sulfite	Concentration of aldehyde-bisulfite by weight.	Lactic acid equivalent to aldehyde titrated	I_2 used	Aldehyde equivalent of bound SO_2 Percent of error from theory by weight
	<i>M</i>	<i>mg</i>	<i>N</i>	
NaHCO_3	8×10^{-2}	180 0	0 1	-0 3
"	5	11 0	0 01	-0 4
" + glycerol	5	11 0	0 01	+0 1 to -0 4
K_2HPO_4 + "	5	11 0	0 01	-0 4
Borax + "	5	11 0	0 01	+0 1 to -0 4
NaHCO_3	2×10^{-3}	4 5	0 002	+1 0
" + glycerol	1 8	4 0	0 01	+1 5
" . . .	4×10^{-4}	0 9	0 002	+0 5
" + glycerol	1	0 225	0 002	+7 0
Borax + glycerol	1	0 225	0 002	+5 6
" + "	2×10^{-5}	0 045	0 002	+4 0 to +8 0

solved to keep the reaction around pH 8.4. Without the protective action of the aldehyde this reaction leads to considerable errors from air oxidation in the titration of pure bisulfite, but, as the data in Table II show, the titration of acetaldehyde-bisulfite (in presence of the excess acid mentioned above) yields approximately correct results with even large amounts of bicarbonate. The simplest plan (under the conditions stated) is merely to add a few tenths to $\frac{1}{2}$ gm. of solid bicarbonate and more if the rate of I_2 consumption becomes slow. When the room temperature is

high and that of the solutions 25° or more, less bicarbonate is needed than when colder; but under the conditions described wide latitude is permissible without significant differences in the result, *provided enough be added to insure the end-point being reached*. To avoid this uncertainty more bicarbonate may be added at the end to test the end-point.

Another equally satisfactory alkali is borax in the presence of an excess of glycerol. The glycerol so increases the strength of boric acid that under the conditions used the mixture provides a suitable reaction (pH about 8) for the rapid liberation of the bound sulfite. The use of borax without glycerol gives too great alkalinity and low results. A suitable amount of borax is the same volume of a saturated solution as the volume of 0.1 N I₂ used in the first stage of the titration, and half that volume of 20 per cent aqueous solution of glycerol.

Sodium or potassium phosphate (B₂HPO₄ 0.6 to 1 cc. of M solution or equivalent for each cc. of 0.1 N I₂) is also satisfactory.

Sodium carbonate alone, and to less extent with glycerol,⁹ gives low results unless the solutions are quite cold; its use is not recommended.

Behavior of Acetone and Formaldehyde.

The combination of bisulfite with acetone is much weaker than with acetaldehyde, and of formaldehyde much stronger. Because of this difference we hoped to be able to titrate acetaldehyde correctly in the presence of acetone and formaldehyde, but this appears to be impracticable. In very dilute solutions a very considerable fraction of the acetone-bisulfite will be dissociated at the first end-point if it be adjusted *slowly*. But the amount of dissociation so depends upon time and temperature that it is

⁹ About one-fourth of the bisulfite is lost during the aeration by volatilization of some SO₂ and by air oxidation to sulfate. This loss is of no consequence so long as enough is left to hold the aldehyde; but to insure such excess a considerable margin is allowed. The loss by oxidation is very markedly decreased and the stock solution is more stable if glycerol be added to the sulfite solution. This device *cannot be used* however because during the aeration, or on standing exposed to air, some of the sulfite becomes bound as though aldehyde were formed. This is probably due to an oxidation of small amounts of glycerol to glyceric aldehyde or other product, the oxidation being induced by the air oxidation of sulfite.

difficult to make quantitative statements about it. It is certain, however, that in the determination of lactic acid in blood or urine the error due to the formation of acetone from oxidation of hydroxybutyric (or citric) acid will be much less than corresponds to the amount of acetone formed. As indicated in Table IV hydroxybutyric acid interferes only to 1 per cent or less of the amount present, and acetone preformed and from acetoacetic acid is removed by preliminary boiling. Except when relatively large amounts are present, it is our impression that the acetone can be neglected without serious error. The aldehyde may be removed and removed by distilling the mixture after adding sodium peroxide, and the acetone determined in the distillate (8). We know of no way to accomplish the reverse operation.

If formaldehyde is present, however, it interferes to a greater extent. At the slight alkalinity resulting from the addition of CO_2 or borax + glycerol, the bound sulfite of formaldehyde is liberated slowly as compared with that of acetaldehyde compound. But when both are present the second end-point is fading and uncertain, and the results are high for acetaldehyde, due to slow dissociation of the formaldehyde compound. On equal amounts of the two aldehydes one may perhaps guess the correct end-point for the acetaldehyde with a plus error of up to 30 per cent. So far as possible substances which give formaldehyde on oxidation must therefore be removed. Sugars appear to be the most common source and these are removed by fermentation and lime (9).

Description of Method.

Solutions.

Sodium Bisulfite.—1 per cent (0.1 M) solution. 5 to 10 cc. (more for large amounts of lactic acid) are used for each determination, and enough water added to cover the beads in the test-tube. There should be an excess of about 5 cc. or more of 0.1 N over the amount required to combine with the aldehyde.

Potassium Permanganate.—Approximate 0.1 N stock solution (10 gm. to liter) diluted by cylinder as needed to 0.01 or 0.002 N.

Sulfuric Acid.—Approximately 10 N (285 cc. of concentrated

H₂SO₄ to liter) containing also 0.5 M or 10 per cent MnSO₄. U
10 cc.

4. *Standard Iodine*.—0.1 N standardized at intervals again
thiosulfate, which in turn is standardized with KH(IO₃)₂. (3.5
gm. of KH(IO₃)₂ to a liter gives a solution which is 0.1 N I₂ whe
reacting with excess KI and H₂SO₄.) The 0.1 N I₂ is diluted t
0.01 or 0.005 N strength, preferably fresh each day, and protectec
from direct sunlight. The 0.1 N I₂ is used for the first stage -^e +⁺
titration, the end-point being accurately adjusted with th
iodine solution (and back titrated if overrun, with thioy
The 0.01 or 0.005 N I₂ is used to titrate the bound sulfite
adding NaHCO₃ or other alkali to liberate it.

5. *Starch Solution*.—5 gm of arrowroot starch suspen
cold water, poured slowly into 500 cc. of boiling water and l
for 20 minutes. After standing the supernatant liquid is dec
for use.

6. *Alkali to Liberate the Bound Sulfite*.—Saturated solutio
solid NaHCO₃·K₂HPO₄ or borax + glycerol may be used ins
but no advantage is gained.

7. *Talcum powder*.

Procedure.

The lactic acid solution (after suitable preliminary treati
for the removal of interfering substances) is placed in a 30
Kjeldahl flask with 10 cc. of the sulfuric acid-manganous su
solution, and sufficient water to bring the volume to about 8
100 cc. is added. A pinch of powdered talcum is added to pro
even boiling. The flask is connected to the condenser, and
suction pump started (see Fig. 1). The flask is heated with
micro burner and the solution kept boiling vigorously. R.
aeration for a minute or 2 into an empty receiving flask (be
adding KMnO₄) removes any volatile bisulfite-binding substan
such as acetone, if any such be present. Then the flame .
moved and the aeration is stopped while another receiving
containing the bisulfite solution is put in place, when the aera
and heating are resumed. (The preliminary distillation may
omitted in case no volatile interfering substances are prese
The permanganate solution is now dropped into the funnel t
at such a rate that the solution is kept colorless or nearly

The oxidation proceeds rapidly and if the permanganate is not added too fast the pink color fades immediately after each drop until most of the lactic acid is gone. Although the manganous salt greatly reduces the danger from excess permanganate it is wise to regulate its addition to the rate of consumption so as to avoid excess. When the pink color begins to fade slowly the addition of permanganate is discontinued for a few moments until the color fades, then resumed slowly until the pink persists for about 1 minute, and manganese dioxide has separated. This occurs about 10 minutes after starting the oxidation. The boiling is continued for 5 minutes more to sweep out all the aldehyde, when the flame is removed, the air current stopped, the receiving flask lowered, and the tower rinsed five to seven times with 5 cc. portions of water. The excess bisulfite is titrated with 0.1 or 0.05 N iodine using starch indicator, and the end-point carefully adjusted to a delicate blue with the dilute iodine to be used in the titration of the bound bisulfite. The bound bisulfite is then set free by adding NaHCO_3 either solid or in solution, and the bound sulfite is titrated with the dilute iodine solution. If in doubt as to the final end-point more bicarbonate is added; if the end-point has been reached the blue color persists after such addition for 15 seconds or longer; otherwise it fades quickly.

A blank should be run on the reagents and should be made so as to include any sulfite-binding material drawn into the apparatus by the air current. The blank will not be high unless the room air contains aldehyde or acetone vapor, as will happen if these substances are used near by. It is wise to exclude the use of all such material from the room and neighboring portions of the building when determinations are run. By slightly modifying the apparatus the ingoing air may be washed through bisulfite and this error avoided, but it is simpler to exclude the sources of all such interfering vapors from the room, and to test their absence by blank determinations on the reagents alone. The titration of the blank is subtracted as a correction before calculating the results. Each cc. of 0.01 N I_2 represents 0.5 cc. of 0.01 M acetaldehyde or lactic acid and is equivalent to 0.45 mg. of lactic acid. In analyses of blood filtrates it is preferable to use 0.002 N I_2 , each cc. of which represents 0.09 mg. of lactic acid.

Analysis of Zinc Lactate.

The accuracy of the method as a whole is indicated by the results of determinations on solutions of pure zinc lactate shown in Table III. Within optimum ranges the extreme variations are about 5 per cent, and the average of more than a hundred determinations on from 0.04 to 180 mg. of lactic acid is 97.4 per cent, the maximum deviations being -10 and +5 per cent.

TABLE III

Summary of Determinations on Pure Zinc Lactate, Showing Minimum, Maximum, and Average Results

Lactic acid in solution oxidized <i>mg</i>	No of analyses	Per cent of recovery		
		Minimum	Maximum	Average
0.045 to 0.5	22	95.0	105.0	98.7
0.5 to 1.0	15	95.8	100.6	97.6
1.0 to 5.0	35	94.0	99.0	96.5
5.0 to 15.0	29	94.5	97.8	96.4
45	2	97.2	99.3	98.3
180	4	89.8	97.0	96.7
				97.4

With amounts above 15 mg more MnSO_4 (20 cc of 0.1 M up to 20 gm) was used, and stronger solutions of bisulfite to collect the aldehyde. With 45 mg of lactic acid the yields with 5 cc and 20 cc of 0.1 M MnSO_4 were 97.2 and 99.3 per cent respectively. With 180 mg the yields with varying amounts of MnSO_4 were the following: with 20 cc M, 89.7 per cent; with 2 gm, 93.7 per cent, with 10 gm, 96.4 per cent; with 20 gm, 97.0 per cent.

Interfering Substances

Many substances, especially hydroxy acids and sugars, as noted by Clausen, may yield bisulfite-binding substances on oxidation. The reflux condenser reduces this effect by allowing only the more volatile products to be aerated over; this is true, however, only when the condenser is kept cool.

In Table IV are listed a number of substances with their yield of bisulfite-binding or iodine-reacting products when analyzed as described above for lactic acid. The results are stated in terms of per cent of the yield realized from the same weight of lactic acid.

TABLE IV.

Maximum Yield of Bisulfite-Binding Substances from Various Compounds.

These data were obtained with apparatus having efficient well cooled condensers. The results in general are higher with less efficient or warm condensers. The results from the oxidation of substance are expressed in per cent of the bound sulfite from an equal weight of lactic acid. 5 to 10 mg. of each substance were analyzed.

None or less than 1 per cent	1 to 5 per cent	10 to 15 per cent
Glycollic aldehyde	Glucose.	Glyceric acid.
Glyoxylic acid.	Galactose.	Malic acid.
Glycol.	Tartaric acid.	
Glyoxal.	Maleic acid.	Greater than 15 per cent
Glycerol	Tyrosine.	Cystine (15 to 22 per cent).
Sodium glycerophosphate.		Rhamnose (25 to 29 per cent).
Succinic acid.	5 to 10 per cent	Citric acid (18 per cent).
Levulinic "	Fructose.	α -Hydroxyisobutyric acid (86 per cent).
Saccharic "	Xylose.	
Glycocol.	Arabinose.	
Alanine *	Dihydroxyacetone.	
Phenylalanine.	Glyceric aldehyde.	
α -Aminobutyric acid.	Fumaric acid.	
Glutamic acid.	Erythritol	
Aspartic "		
Glycosamine.		
Leucine.		
Urea		
Uric acid		
Allantoin.		
Creatine		
Creatinine.		
Hippuric acid.		
Barbituric acid.		
Glycollic acid.		
Pyruvic acid.		
Methylglyoxal †		
Malonic acid.		
Menthol glycuronic acid.		
Mucic acid		
Arabonic acid †		
β -Hydroxybutyric acid §		

* This was true only with a well cooled condenser. With a warm condenser a yield of 4.6 per cent was obtained

† Solutions of methylglyoxal always contain some preformed lactic acid. This amount is identical before and after oxidation of the glyoxal by alkaline H_2O_2 .

‡ Less than 0.1 per cent.

§ From *l*-calcium zinc salt, isolated from diabetic urine, the yield varied. Two preparations yielded 0.7 and 10.4 per cent respectively. The presence of lactate as an impurity is not unlikely. The synthetic *dl*-salt gave only 0.8 per cent

|| The conversion into acetone was quantitative

The figures permit convenient estimate of the degree of interference of these substances if present in solutions being analyzed for lactic acid. The results were obtained under conditions duplicating those described for the lactic acid determination with apparatus having efficient, well cooled condensers. With warm condensers the interference is in general greater. The data indicate that the main nitrogenous constituents of urine and of muscle extracts yield, under the conditions, little or no products which might be mistaken for lactic acid. Fructose, pentoses, and the trioses have the effect of less than 10 per cent of the same amount of lactic acid, glucose less than 5 per cent, while cystine, citric, and hydroxyisobutyric acid have much greater effect. Glycol, glycerol, glyoxal, β -hydroxybutyric acid, and pyruvic acid have practically no effect. The fact that the last named substance yields no acetaldehyde indicates that pyruvic acid is not, as might be expected, an intermediate between lactic acid and aldehyde under these conditions. So far as most of the substances listed are concerned it seems unlikely that they would lead to grossly erroneous results for lactic acid with our method unless present in relatively large amounts. It is nevertheless desirable that interfering substances be removed whenever means are available, since the presence of other oxidizable substances uses up more permanganate, prolongs the oxidation, and leads to uncertainty as to when the oxidation of the lactic acid is finished.

The interfering substance formed from oxidation of the sugars is probably formaldehyde, since Rosenthaler, Klein (10), and others observed its formation under similar conditions. The character of the last end-point in the titrations gives an indication of the presence or absence of substances other than acetaldehyde; and with solutions from the oxidation of sugars it is often slow and fading, just as is the case when formaldehyde is known to be present.

Removal of Interfering Substances.

The removal of volatile substances such as acetone preformed and from acetoacetic acid is accomplished by preliminary boiling and aeration as described under "Procedure." We can confirm the statement of Clausen and others that lactic acid is not precipitated or lost in the treatment with CuSO_4 and milk of lime for

removal of sugar, nor in the Folin-Wu precipitation of proteins by tungstic acid. Blood and tissue extracts are therefore first precipitated with one or both of these procedures and the filtrates are analyzed for lactic acid.

The results with tungstic acid filtrates, with and without subsequent copper hydroxide treatment, differ but little, though when sugar is not removed the oxidation proceeds more slowly and less smoothly and the end-point in the titration is less sharp.

One of the best means of removing proteins and other nitrogenous compounds from blood and tissue extracts is the Patein-Dufau precipitation with acid mercuric nitrate and bicarbonate; but this treatment unfortunately causes considerable loss of lactic acid. The Schenk treatment with HgCl_2 in acid solution does not cause loss. This aspect of the pretreatment of blood and tissue extracts for lactic acid determination has been specially studied by Dr. E. Ronzoni, and will be considered by her in a forthcoming paper.

In dealing with sugar solutions, such as after conversion of sugar to lactic acid by alkali, we find but little difference between results for lactic acid in the filtrates from copper hydroxide treatment and in solutions of the ether-extracted material. In both cases the results appear to represent fairly accurately the true lactic acid present, and either method of pretreatment is therefore satisfactory. Data on this point will be included in a later paper by one of us (F.).

SUMMARY.

A procedure is described for the determination of lactic acid, based on oxidation to acetaldehyde and titration of sulfite bound by the aldehyde. The steps in the process were separately investigated. The use of manganous salt markedly increases the rate of oxidation and also increases the yield of aldehyde. The manner of its action is discussed. The effect of manganese salts together with the form of apparatus used for the oxidation and collection of aldehyde simplifies and shortens the method as well as makes the results more nearly quantitative and less variable. The conditions for the Clausen titration of bound sulfite were studied and its accuracy established. The extent of interference of about 50 substances was determined.

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A CONDENSER UNIT FOR USE IN THE DETERMINATION OF LACTIC ACID.

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The apparatus shown diagrammatically in Fig. 1 illustrates an optional variation in the device described by Friedemann, Cotonio, and Shaffer¹ for use in the determination of lactic acid. It possesses an advantage by permitting the apparatus to be very compactly and conveniently arranged. The chief feature of the variation is the condenser unit made of glass tubing wound into a spiral which has an external diameter of about 23 mm. This spiral condenser fits into the neck of a 500 cc. Kjeldahl flask and serves the same function as the Hopkins' type of condenser. When the spiral condenser is used, a single unit of the apparatus can be set up in a space 20 cm. in width, 35 cm. in depth, and 75 cm. in height. Multiple units can be relatively more compactly arranged.

The coil should be made of approximately 5 mm. Pyrex tubing and the tube through which the permanganate solution is introduced can be 4 mm. tubing. The coil can be formed by winding the heated tubing on a mandrel 15 cm. long and 1.2 cm. in diameter at the small end and 1.4 cm. in diameter at the large end. The mandrel should be made of thin sheet iron (0.25 mm. in thickness) so that it will be sufficiently flexible to be withdrawn easily from the coil. After the permanganate delivery tube and coil have been fitted into the stopper they should be heated, at some point below the stopper, enough to soften the glass and relieve strains.

The vapor outlet tube should have a bore of approximately 7 mm. and may be conducted in any convenient direction to the absorption flask and tower. In our apparatus this tube is 20

¹ Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, lxxiii, 335.

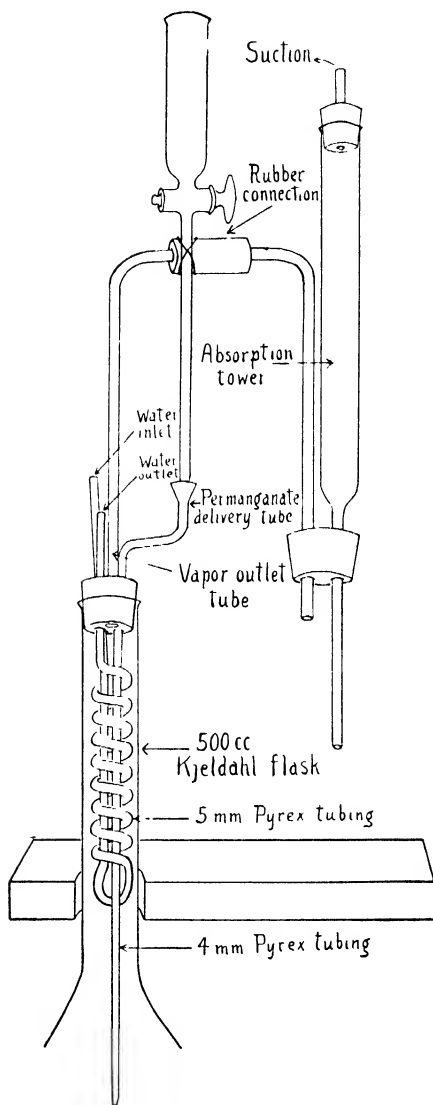


FIG. 1.

cm. long and has a right angle bend 6 cm. long at the top. A similar tube is connected to the first with a short piece of thick walled rubber tubing and leads downward through the stopper of the absorption flask. The rubber connection is supported by a clamp and acts as a pivot for the condenser unit, thereby enabling the Kjeldahl flask to be tilted upward and removed easily. The dropping funnel is supported by wiring it to the rubber connection. It is then adjusted so that its tip articulates with the small funnel of the permanganate delivery tube, and since both the condenser unit and the dropping funnel then have a common pivot, the adjustment is permanent.

A wooden shelf made from 1×8 inch lumber serves the double purpose of supporting the absorption flask (when it is disconnected) and holding the Kjeldahl flask outward at an angle of about 20° to the perpendicular. A notch 3.5 cm. square is cut in the front side of the shelf and the lower portion of the neck of the flask rests in this notch. No clamp is needed to support the flask as it is amply held by the stopper of the condenser unit.

THE CHOLESTEROL CONTENT OF HAIR, WOOL, AND FEATHERS.

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It has become evident as a result of previous studies (1, 2) that a considerable difference exists between the cutaneous and subcutaneous lipids of man; the latter were reported to contain only 0.25 per cent of cholesterol and but mere traces of the phospholipids, whereas the former were found to have as much as 20 per cent of the sterol and 2.5 per cent of the phospholipid. No difference between the fatty acid make-up of the two types of lipids was, however, demonstrable.

The present communication is the report of analyses made on the lipids of human hair, of the hair of various animals, and of the feathers of several birds. The lipids were removed from the samples according to a method previously described (2) for the extraction of them from the skin, which consisted in extracting the material first for 48 hours with the hot vapors of absolute alcohol, with chloroform vapors for 18 hours, then with ethyl ether for a similar period, and finally again with absolute alcohol for 18 hours. The percentage of total lipids was then determined as described in the previous paper (2). The digitonin method was again employed to determine the free and the combined cholesterol.

The data obtained as the result of the analyses of the various samples of hair are grouped in Table I. For the sake of comparison, the averages of the results previously reported for the cutaneous and subcutaneous lipids are also included in the table. It is evident from the results which are referred to, that the lipids of the hair contain amounts of cholesterol that exceed those present in the subcutaneous lipids of man, but on the other hand, it is also

TABLE I

Percentage of Lipids and Cholesterol in the Hair of Man and Other Animals.

Source of sample			Free cholesterol		Total cholesterol	
			In sample	In lipids	In sample	In lipids
			per cent	per cent	per cent	per cent
Human adult (P).			8.4	0.07	0.82	1.2
" " (H).			6.6	0.09	1.40	1.4
" " (E)			4.3		0.23	5.4
" " (S)			6.9		0.17	2.5
" " (RH)			4.5		0.12	2.6
Children	Years	Individuals				
"	2		3.5		0.37	10.6
"	4		2.9	0.19	6.50	10.1
"	2-5	4	3.2		0.31	9.6
"	7	4	4.2	0.29	7.00	10.1
"	10-12	5	3.6	0.20	6.50	8.7
"	10-13	5	3.9	3	0.45	11.8
Rabbit*169			1.5	0.53	34.00	0.56
" 176			1.4	0.56	40.10	0.58
" 171			1.5	0.45	31.10	0.53
" 177			1.7	0.54	31.60	0.58
" 200			1.6	0.58	38.20	0.61
Rat			4.3	0.41	9.6	0.57
Cat			5.3	0.46	8.7	0.55
Dog.			1.8			0.56
Subcutaneous lipids (1)						0.2
Human skin (2)					0.93	19.6

* The blood cholesterol content for the five rabbits given in the same order as in the table and expressed as mg per 100 cc was 178, 114, 243, 200, and 118

true that with the exception of rabbit¹ and dog hair, less of the sterol occurs in the lipids of the hair than in the cutaneous lipids of man. That the cholesterol found in human hair does not owe its presence there to the possibility of having been rubbed on the

¹ The samples of rabbit hair were obtained from the Department of Medicine, through the courtesy of Dr. L. H. Newburgh, in whose laboratory the blood cholesterol determinations of these rabbits were made.

hair in the form of cosmetics was made certain from inquiries made of the individuals from whom the samples of hair were obtained.

As a result of the analyses of human hair it is evident that there is more cholesterol present in the lipids removed from the hair of children than in those extracted from adult hair. Expressed as per cent of the total lipids the average for adults is 2.6 per cent as compared with 10.1 per cent, the average figure for children, and when the results are expressed as per cent of the hair itself it is to be seen that children's hair contains 0.36 per cent of the sterol while adult hair has only 0.15 per cent. The percentage of cholesterol in an oil such as cod liver oil is said to vary from 0.5 per cent to 25 per cent. It is evident, therefore, that the lipids in children's hair have at least 4 times as much of this non-saponifiable lipid as does this particular fish oil, and it is likewise true that there is more cholesterol in the hair of children than in their own blood. Most of the samples were secured from the University Hospital,² and since sufficient amounts were not available for separate analyses these samples were consequently combined to make up the groups indicated in Table I.

The lipids in the hair of the rabbit and the dog are distinctive in that nearly one-third of them is present as cholesterol. The first sample of rabbit hair analyzed was obtained from an animal having a very high amount of blood cholesterol, and this fact was at once thought to be a factor contributing to the marked sterol content of the lipids in that hair. Subsequent analyses, however, proved that that was not the case, for of the other animals whose hair was analyzed, two, Rabbits 176 and 200, had a normal blood cholesterol, while two others, Rabbits 171 and 177, showed cholesterolemia (see Table I). The cholesterol contents of the lipids in the hair of the first two rabbits just referred to were 41.2 per cent and 38.7 per cent, as compared with 35.0 per cent and 33.9 per cent for the latter two. It is evident, therefore, that the extremely high cholesterol content of the lipids in the hair of Rabbit 169 is not to be explained by the high blood cholesterol content of this animal. In spite of this unusually and unex-

² These samples were obtained through the courtesy of Dr. Udo J. Wile of the Department of Dermatology and Syphilology and Dr. D. M. Cowie of the Department of Pediatrics.

pectedly high amount of the sterol in the lipids of rabbit and dog hair, the hair of these animals itself contains no more cholesterol than does the hair of the rat or the cat, the reason being that the total lipid content of dog and rabbit hair is very low, while that

TABLE II
Percentage of Lipids and Cholesterol in Wool and Feathers.

Source of sample	Total lipids in sample	Free cholesterol		Total cholesterol	
		In sample	In lipids	In sample	In lipids
	per cent	per cent	per cent	per cent	per cent
Sheep 1 ..	8.9	0.37	4.2	1.11	12.5
" 2 .	10.1	0.42	4.1	0.63	6.3
Lamb 3	12.7	0.56	4.5	1.09	8.6
" 5a	8.9	0.44	5.0	0.63	7.0
" 5b	13.7	0.49	3.6	0.63	4.6
" 5c	9.4	0.35	3.7	0.75	8.0
" 5d	13.2	0.64	4.9	1.05	7.9
" 5e	9.4	0.52	5.6	0.63	6.7
Turkey feathers (A)	1.9			0.26	14.1
" " (B)	1.1			0.28	25.8
Goose " (C)	1.9			0.27	13.6
Duck " (D)	3.2	0.18	5.4	0.29	9.4
Quills from (A)	2.2			0.37	17.2
" " (C)	4.6			0.30	6.7
" " (D)	3.6			0.38	10.6

Sheep 1, full fleece animal, is mother of Lamb 3

" 2, " " " " " 5.

Lamb 3, " " " 18 days old when sample was taken

" 5a, " " " 55 " " " " "

" 5b, sample was taken from same place as Sample 5a, 4 wks later

" 5c, full fleece, taken at same time as Sample 5b.

" 5d, " " cut 5 wks later.

" 5e, " " taken at same time as Sample 5d but from another place

of the hair of the other two animals is rather high in comparison to that of the dog and rabbit. Thus, while the cholesterol content of the lipids removed from the hair of the cat and the rat is only 10.3 and 13.3 per cent respectively, the amount of lipids in their

hair is such that the percentages of the substance in question, calculated on the basis of the hair, are 0.57 and 0.55 per cent respectively, as compared with 0.57 and 0.56 per cent for the rabbit and dog, respectively.

Contrary to expectations, wool fat was not found to contain notably larger amounts of cholesterol than the other lipids studied. A comparison of the results in Table I with some of those in Table II shows that with the exception of the wool fat obtained from Sheep 1³ there is more cholesterol in the lipids extracted from the hair of the various animals than in those obtained from the wool. While it is true that the lipids of adult human hair are poorer in their cholesterol content than any of the samples of wool fat analyzed, it is also quite evident that the hair of children contains lipids that are richer in this respect than were seven out of the eight samples of wool examined. On the other hand, it is readily seen that the wool itself contains more of the sterol than does any sample of hair analyzed. This is brought about by the fact that the percentage of total lipids in the wool far exceeds that found for any of the samples of hair. A further study of Table II shows that there is a considerable variation between some of the samples analyzed. This was rather to be expected in the light of the findings of Herbig (3) who found that the non-saponifiable fraction of different samples of wool, calculated as per cent of the wool, ranged from 2.6 to 7.3 per cent. The first four samples of wool in Table II represent the full length of wool and were clipped from the animals on the same day. The variation expressed as per cent of the total lipids is from 6.3 to 12.5 per cent. Although sufficient data have not been secured to explain these differences, there are certain facts to which attention should be called at this time. While it may be of no significance, it is nevertheless true that the wool of Sheep 1 and its lamb (Lamb 3) contained more cholesterol than did the wool of Sheep 2 and its lamb (Lamb 5). There may thus be some hereditary factor that determines the amount of cholesterol in the wool. Lamb 5 was shorn on two later dates so that five samples in all were secured from that

³ The different samples of wool were secured from the Michigan Agricultural Experiment Station, through the cooperation of Dr. C. S. Robinson and Prof. G. A. Brown.

animal. A description of these samples is included in Table II. The variation for this particular animal, expressed as per cent of the total lipids, is from 4.6 to 8.0 per cent. The lowest figure is the one for Sample 5b, which was clipped from the same place where the animal had been shorn a month previously. The percentage of the total lipids in that sample is, however, sufficiently high enough to make the cholesterol content of the wool itself come up to the same level found at the first cutting. Were it not for the fact that the amount of cholesterol in Sample 5e is practically the same as that of Samples 5a and 5b, it would appear as if there is a progressive increase of the sterol as the animal becomes older, since the percentage of cholesterol is greater in Sample 5c than in Samples 5a or 5b, and furthermore because the amount present in Sample 5d is more than in any of the other samples. The differences between the various samples of wool can also be explained merely by assuming that those samples having a higher content of cholesterol do so for the reason that they contain more of the wool that had been clipped near the roots than do samples having less of the sterol. Such a view would be in line with the report of Lifschutz (4) who has recently come to the conclusion that the concentration of this non-saponifiable lipid is greater near the roots than at the tips of the wool. He gives no actual figures, but merely bases his conclusion on the fact that wool clipped close to the roots contains lipids that give a more intense color reaction than does wool clipped near the tips.

The cholesterol content of the lipids removed from feathers is also variable. As is shown in Table II, the lowest figure (9.4 per cent) was that obtained from a sample of duck feathers, and the highest (25.8 per cent) from turkey feathers. The total lipid content of feathers has, however, a tendency to be inversely proportional to the amount of cholesterol present in these lipids, and when this is taken into consideration it will readily be noted that the amount of cholesterol present in the different feathers themselves is remarkably constant. Calculated as per cent of the feathers, the range is only from 0.26 to 0.29 per cent. The quills of these same feathers likewise contain almost constant amounts of cholesterol, in spite of the fact that the percentage of the sterol expressed as per cent of the total lipids varies. Thus, while the variation just referred to is from 6.7 to 17.2 per cent, the range

is only from 0.30 to 0.37 when the calculation is made as percentage of the quills.

SUMMARY.

1. The cholesterol content of the lipids in human hair as determined by the digitonin method exceeds that previously reported for the lipids of the subcutaneous layers of man. It is, however, not as high as that reported for the lipids in human skin.

2. The lipids of rabbit and dog are distinctive, in that practically one-third of them is present as cholesterol, whereas only 13.3 per cent of the lipids extracted from rat hair, and but 10.3 per cent of them in cat hair, exist in that form. Calculated on the basis of the hair itself the percentages for the rat and the cat are 0.57 and 0.55 per cent respectively, as compared with 0.57 and 0.56 per cent for the rabbit and dog respectively.

3. The lipids of wool cannot be characterized by their high cholesterol content, since the largest amount in any of the wools analyzed is only 12.5 per cent of the total lipids, while as much as 41.0 per cent is present in the lipids of rabbit hair. The wool itself, however, contains more of the sterol than do any of the samples of hair, the variation for wool expressed as per cent of the wool being from 0.63 to 1.11 per cent.

4. The cholesterol content of the lipids removed from feathers is also variable, the lowest figure (9.4 per cent of the total lipids) is that obtained for duck feathers, and the highest (25.8 per cent) is that found for turkey feathers. Calculated on the basis of the feathers themselves, the range is only from 0.26 to 0.29 per cent.

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ON THE ISOELECTRIC PRECIPITATION OF PEPSIN.

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In attempting to separate pepsin from the glandular mucosa of hog stomachs we were confronted with four main problems.

The purification process must be simple and brief in order to retain the original properties of the enzyme which is unstable in solution and sensitive to chemical treatment. Peptic hydrolysis of the glandular mucosa must be avoided as far as possible. If the enzyme is permitted to exercise its liquefying properties, in presence of acid and at body temperature, on the tissue in which it is produced it loses its identity to such an extent that it can no longer be separated from the solution by adjustment of the hydrogen ion concentration. This observation was made after several vain attempts to isolate the enzyme from digested linings. The soluble blood and tissue salts must be removed as they act as solvents for the enzyme and interfere with its precipitation at the isoelectric point. The mucin which abounds in the linings must be eliminated from the solution before the enzyme can be precipitated successfully. If mucin is present it is carried down with the enzyme mechanically and constitutes a troublesome contaminating factor.

After considerable experimental work the following procedure was devised. In order to reduce hydrolysis to a minimum the entire extraction process was carried out in a specially constructed chill room maintained at 0°C. All ingredients employed were properly chilled to the same temperature before use.

The pepsin-producing glandular layers from hog stomachs are dissected out a few minutes after the animals are slaughtered and transferred to the chill room. Here they are trimmed free from fat and muscular tissue, washed and brushed in ice water until all adherent mucin is removed, and finely minced. The fresh lin-

ings usually contain about 85 per cent of moisture. To each kilo of hash is added while stirring, 400 cc. of distilled water containing a suitable amount of HCl, usually 2 per cent. This mixture is set aside for some hours or overnight to facilitate extraction. Acetone is next added until the mixture contains a volume equal to its total water content. This solvent mixture extracts the pepsin, together with soluble cell and blood constituents and acid protein salts formed during the process, leaving the tissues and mucin undissolved. The liquid is strained off and filtered. The reaction of the clear extract should lie between pH 3.4 and 3.6. This is an important point. If an excess of acid is employed for extraction the yield of recoverable pepsin will be materially decreased and the amount of acetone necessary to produce precipitation increased. By raising the acetone content of the liquid to 75 per cent the pepsin fraction is thrown down as a whitish clingy sediment. Precipitation is rapid and the supernatant liquid containing most of the objectionable impurities may be decanted off after a few minutes standing. The precipitate is centrifuged to remove the balance of the solvent and desiccated *in vacuo* at low temperature.

The dry product resembles commercial pepsin in appearance but differs in character by being only partly soluble in distilled water. It is soluble in acidulated water.

A clear filtered solution of such pepsin in acidulated (HCl) water is suitable for precipitation. This may be accomplished by soluble bases such as ammonia, the bicarbonates, carbonates, or hydroxides of sodium and potassium, etc., but the largest and best yields are obtained by dialysis. The reason for this is readily explained. Alkalies even in dilute form are destructive on pepsin and the mineral salts formed tend to keep the enzyme in solution at its isoelectric point. Dialysis is a mild treatment where there is a gradual decrease of acidity without salt formation. Precipitation occurs at hydrogen ion concentrations ranging between 2.4 and 3.85 on the pH scale. The solution begins to get opaque at pH 2.2 but no distinct precipitation occurs until the 2.4 point is reached. In order to obtain definite information regarding the amount and nature of the precipitate formed at different stages within this wide zone, fractional precipitation was carried out.

The products employed for analysis were obtained by dissolving the acetone-precipitated pepsin in acidulated water and adjusting

the reaction to a pH of 1.8 to 2.0. After filtration this liquid was dialyzed rapidly against cold running water (5–10°C.). The first fraction represents precipitates formed at pH 2.4 to 2.5, the second at pH 2.5 to 3.0, and the third at pH 3.0 to 3.85. The various fractions were separated from the solution by centrifuging at high speed, washed in 20 volumes of distilled water, and desiccated *in vacuo* at room temperature. They appear as white granular powders nearly insoluble in distilled water. They are freely soluble in 0.3 per cent hydrochloric acid at body temperature, slowly but clearly so at room temperature, and only to the extent of about 1 per cent at 0°C. The dialyzed liquid containing the non-precipitable and non-dialyzable pepsin-protein fractions was likewise dried at low temperature and negative pressure. This is as far as we were able to purify the enzyme fractions and show gain in proteolytic activity. Repeated dialysis or precipitation did not improve the quality but invariably lowered the quantity of the products.

Twenty-six batches from 5 to 10 kilos of linings representing 40 to 80 hogs respectively were made with a week's interval from September, 1926, to March, 1927. The yield of acetone precipitate ranged from 7 gm. testing 1:50,000 to 6 gm. testing 1:20,000 per kilo of fresh linings. The purified enzyme fractions varied from 1.6 to 0.6 gm. of the pH 2.4 to 2.5 fraction, from 0.9 to 0.4 gm. of the pH 2.5 to 3.0 fraction, and from 0.6 to 0.3 gm. of the pH 3.0 to 3.85 fraction per kilo of fresh material. The proteolytic activity of the pH 2.4 to 2.5 fraction varied from 1:70,000 to 1:45,000 with an average value for the 6 months of 1:52,500. The pH 2.5 to 3.0 and 3.0 to 3.85 fractions were of lower but more constant activity. Here the digestive power ran from 1:50,000 to 1:42,500 and the average was 1:45,000. The amount of pepsin in the individual mucosa varies considerably. Pale, thin linings from fatigued animals are low in proteolytic properties. Besides the individual fluctuation, a seasonal variation also exists. The best yields of the highest testing pepsin were obtained in the fall and the lowest toward spring.

Pekelharing and Ringer (1) in 1911 reported the isoelectric point (Flockungsoptimum) of a single sample of highly purified pepsin made from hog stomachs at pH 3.32. This figure falls within the zone reported in this paper but it does not coincide with our pre-

precipitation optimum. The proteolytic activity and chemical analysis of this sample were not furnished.

Normal fresh hog stomach linings are acid in reaction and contain some activated pepsin. Most of the enzyme, however, is imbedded in the cells and in the native insoluble form. When the acid and water are added to the chilled and minced linings in the extraction process the formation of acid protein takes place. Only a limited amount of activated pepsin is formed since the enzyme is sparingly and slowly soluble in acid water at 0°C. As the acid hydrolysis proceeds the hydrogen ion concentration of the mixture decreases and eventually reaches into the isoelectric zone for the enzyme protein. At this stage the enzyme is not activated but dissolved unchanged into the mixture. It is at the lower end of the isoelectric zone, *i. e.* pH 3.4 to 3.6, that the 50:50 acetone water extraction is made. When the acetone content subsequently is increased to 75 per cent the pepsin fraction becomes insoluble and precipitates out. This fraction is a mixture of native, activated, and protein-combined pepsin. In order to redissolve this precipitate in water it becomes necessary to bring the reaction of the solution on the acid side of the isoelectric zone for the enzyme; *i. e.*, above a pH value of 2.4. Here the acid salt of the enzyme protein is actually formed. When the acid is removed by dialysis or neutralized by alkalis the enzyme protein again becomes insoluble and separates out. While in solution the enzyme has been in intimate contact with products of hydrolysis of protein, etc. A single isoelectric precipitation and washing with distilled water is hardly adequate treatment to rid the enzyme of all traces of foreign substances. It is, however, at this stage so highly sensitive that further purification results in lowered activity, indicating changes in the protein molecule.

The three enzyme fractions are very unstable in solution on the acid side of the isoelectric zone. If we make a 1 per cent solution of any of the fractions in acidulated water (0.3 per cent HCl) and keep it at 52°C., the optimum hydrogen ion concentration and temperature for peptic hydrolysis, for 2½ hours without adding any protein, the solution loses 90 per cent of its proteolytic activity.

In Table I are given the chemical analyses of composite samples of the acetone precipitate, the various enzyme fractions, and the dialysate. The acetone precipitate contains all the chlorine in

'ABI
Properties of Composite Samples of Pepsin in Various Stages of Purity.

	Moisture.	Ash	Total nitrogen	Total phosphoric acid as P ₂ O ₅	Organic combined chlorine as Cl	Inorganic chlorine as NaCl	Proteolytic power, U S P assay.		
							Maximum.	Minimum	Average.
Acetone precipitate.	per cent 6 60	per cent 1 92	per cent 13 52	per cent 2 72	per cent 1 82	per cent None.	1 50,000	1 20,000	1 30,000
Enzyme fraction precipitated at:									
pH 2.4-2 5	4 40	0 023	14 21	0 57	0 09	"	1 70,000	1 45,000	1 52,500
" 2 5-3 0	4 50	0 025	14 20	0 55	0 09	"	1 50,000	1 42,500	1 45,000
" 3 0-3 85	4 90	0 025	14 22	0 56	0 09	"	1 50,000	1 42,500	1 45,000
Dialysate left after enzyme precipitation.	5 30	1 31	13 33	2 52	2 70	"	1 18,500	1 7,500	1 15,000

organic combination. The ash consists chiefly of calcium, phosphorus, and a small amount of iron. The chemical composition of the three enzyme fractions seems to be identical and it is consequently the same protein which constitutes the entire enzyme precipitate. The very low ash content shows that this protein is quite pure. More detailed work on the nature of the enzyme protein is in progress and will be reported later. The last column gives the data on the composite dry mother liquors from the enzyme precipitates. This product contains the basic pepsin-protein combinations. The proteolytic activity ranged from 1:18,500 to 1:7500 with an average value of 1:15,000. It is freely and completely soluble in distilled water and far more stable in solution than the acid salt of the enzyme. Its chemical composition is very similar to high grade commercial pepsin of equal strength made by the regular digestion and purification processes.

Northrop (2) has pointed out that pepsin digestion is a chemical reaction in which the pepsin as well as the protein takes part. This we have found to be the true state of affairs. It will be seen from the perspective presented in this communication that the process of peptic hydrolysis is a progressive one in which the enzyme combines with other protein and gradually loses its identity and physiological characteristics. When the pepsin-protein-split products reach the stage where they are able to diffuse through parchment or animal membranes they are physiologically inert. The reaction goes on in the presence of sufficient acid at 0°C. as well as at body temperature, but the rate of hydrolysis is many times greater at the higher temperature. It was only by taking advantage of the slow reaction at low temperature and by careful study of the solubility factors and close control of the hydrogen ion concentrations that it became possible to obtain a certain amount of the enzyme in comparatively pure form.

The analytical work was carried out according to the Association of Official Agricultural Chemists methods (3), and the proteolytic activity determined by the United States Pharmacopœia assay (4).

CONCLUSIONS.

A method of isolating the peptic enzyme directly from the gastric mucosa is described and discussed in detail.

The purified enzyme is a nearly ash-free protein quite insoluble in distilled water, but clearly and completely soluble in acidulated water. Its isoelectric zone lies between 2.4 and 3.85 on the pH scale. Fractionation shows the formation of the most active precipitates at the highest hydrogen ion concentration within this wide zone.

The proteolytic activity of the fraction precipitated at pH 2.4 to 2.5 ranges from 1:70,000 to 1:45,000 U.S.P. assay, depending on the condition of the glandular mucosa and the season. The most active preparations were obtained during the fall months and the least active toward spring.

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ABSENCE OF STRATIFICATION AND RAPIDITY OF MIXING OF CARBON DIOXIDE IN AIR SAMPLES.

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A problem in metabolism studies which are dependent upon the analysis of gas samples is the question of adequate mixture of the sample and the possibility of settling of the carbon dioxide into the lower portion of the sampler, if the sample is allowed to stand for hours or days before analysis. Theoretically, it is believed that the differences in the specific gravity of gases may cause stratification of the gases, and when there is a possibility of varying amounts of carbon dioxide in different portions of the sample, the lower diffusion rate of carbon dioxide is supposed to play the rôle in the inadequacy of mixture. Although we had been reasonably certain that stratification does not occur in the sampling of gases for analysis, a recent discussion in European laboratories has raised the question anew and further evidence which we have secured in this connection seems worthy of record.

The following experiments were carried out to gain information on these two points: A sampling tube, 55 cm. long and 5 cm. in outside diameter, with a capacity of 1040 cc. and with stop-cocks at the ends having outlets at end of stopper, was set up and filled with dry air to which was then added a small amount of carbon dioxide. The sample was thoroughly mixed and the sampler connected to the gas analysis apparatus devised in the Nutrition Laboratory (Carpenter, 1923¹). The gas was analyzed immediately and on successive days until all the sample was used. The results are shown in Table I. The **only** change in the percentage of carbon dioxide from the beginning to the end is a lowering.

¹ Carpenter, T. M., *J. Metabol. Research*, 1923, iv, 1.

The last portions of the samples contained no more carbon dioxide than the earlier portions. In other words, there was no indication of stratification on standing. The lowering of the percentage was probably due to traces of moisture on the walls of the sampler.

The rapidity of diffusion of carbon dioxide in a mixture of gases

TABLE I

Analyses of Mixtures of Air and Carbon Dioxide Showing Absence of Stratification of Carbon Dioxide in Air Samples

Mixture 1		Mixture 2		Mixture 3	
Analysis on	Carbon dioxide	Analysis on	Carbon dioxide	Analysis on	Carbon dioxide
1927	per cent	1927	per cent	1927	per cent
Jan 26, p m	1 397	Feb 7	0 539	Feb 18, a m.	1 355
" 26 "	1 383	" 7	0 538	" 18, p m	1 342
" 26 "	1 398	" 8	0 527	" 19, a m	1 350
" 27 "	1 396	" 8	0 528	" 19, p m	1 353
" 28	1 394	" 8	0 525	" 21, a m.	1 345
" 29	1 390	" 9	0 521	" 21 "	1 346
" 31	1 389	" 9	0 522		
		" 9	0 522		
		" 10	0 520		
		" 10	0 517		
		" 10	0 517		

TABLE II

Analyses of Mixtures of Air and Carbon Dioxide Showing Rapidity of Diffusion of Carbon Dioxide (Per Cent of Carbon Dioxide)

Sample prepared Feb 11, 1927, 9 49 a m		Sample prepared Feb 11, 1927, 1 48 p m		Sample prepared Feb 17, 1927, 9 20 a m		Sample prepared Feb 17, 1927, 1 25 p m	
Analysis at		Analysis at		Analysis at		Analysis at	
10 00 a m.	0 996	2 00 p m	0 332	9 30 a m	0 713	1 30 p m	0 109
10 16 "	1 108	2 25 "	1 115	10 10 "	1 126	1 50 "	0 784
10 25 "	1 111	2 45 "	1 113	10 40 "	1 138	2 50 "	0 859
12 10 p m	1 111			11 30 "	1 135	3 20 "	0 855

was also determined in the following way: The same sampler was used and filled with room air. A portion of pure carbon dioxide was introduced into the lower stop-cock from a syringe bulb or gas-sampling tube. After the cocks were closed at each end of the tube, the gas was put under slight pressure by carefully admit-

ting mercury from a leveling bulb connected to the lower end of the sampling tube. Analyses were made on this sample successively until the gas had reached constant composition. The results of several series are shown in Table II. In the first two series the samples were drawn into the gas analysis apparatus by the washing method, in which several portions are drawn into the gas analysis apparatus and subsequently rejected before the final sample is drawn for analysis. The two series of 9.20 a.m. and 1.25 p.m., February 17, were carried out by the displacement method in which the air connections were filled with mercury and, by reversing the upper cock, air was drawn into the analysis apparatus with the least amount of mixing of air in the container. In the first two series complete mixture was reached in 36 and 37 minutes respectively, and in the second two it was reached in 1 hour and 20 minutes and 1 hour and 25 minutes, respectively. The results indicate clearly that the diffusion of carbon dioxide is rapid and complete, even when at first the different portions of an air sample vary in composition, the upper portion containing practically no carbon dioxide and the lower portion containing pure carbon dioxide.

These two sets of experiments show that once a homogeneous sample of air is drawn into the container, it may be analyzed subsequently at any time without mechanical shaking and without fear of inadequate mixing.

SUMMARY.

Experiments were made to determine whether stratification of carbon dioxide takes place in a sample of air when left undisturbed for several days. The results show no evidence of stratification. The rapidity of mixture of carbon dioxide with air was also determined, and the diffusion was found to be rapid and complete even under extreme conditions.

INFLUENCE OF INTENSE X-RAY AND γ -RAY RADIATION ON CHOLESTEROL.

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(Received for publication, March 8, 1927)

Having available at this Institute the means of delivering massive x-ray and γ -ray doses it was decided to follow the changes which this intense radiation might induce in certain organic compounds. Cholesterol was selected for this first study because of its wide distribution in the body and its physiological activity there.

Some work has been done in the field. Hess and Weinstock (1) report that x-rays do not produce an antirachitic change in cholesterol. Roffo (2) claims that 0.02 per cent cholesterol solution in CHCl_3 or C_6H_6 when subjected to rays from an x-ray tube (200 kilovolts 4 milliamperes) for 1 hour without filter gave but traces of cholesterol with the Grigaut test.

The Pfanstiehl cholesterol used in these experiments was dissolved in chloroform or absolute alcohol to the extent of 0.4774 gm. in 25 cc. 15 cc. portions were sealed in glass test-tubes and placed at a distance of 70 cm. from the water-cooled target of a Coolidge x-ray tube. As controls similar tubes were inserted into lead containers and placed at the same distance from the x-ray tube for the same length of time. The lead was sufficiently thick to stop the x-rays.

The x-ray tube was activated under the following conditions: voltage 200,000, current through the tube 30 milliamperes. The radiation was unfiltered. Duration of radiation varied between 48 minutes and 26 hours; the exact time is given in Table I.

Cholesterol solutions were also irradiated by the γ -rays from radium emanation. In this group of experiments the glass tube containing the radon is inserted into a metal container, the walls of which are $\frac{1}{2}$ mm. gold surrounded by $\frac{1}{2}$ mm. brass, in order to

absorb the β particles. This container is immersed in the solution to be radiated.

The solutions, after subjection to the radiation were examined in three ways: (1) chemically, (2) by means of the polariscope, and (3) in the ultra-violet spectroscope.

Fig. 1 shows the absorption spectra curves of cholesterol before and after x-ray radiation. With increasing x-ray dosage the curve moves toward the longer wave-lengths, indicating increased ab-

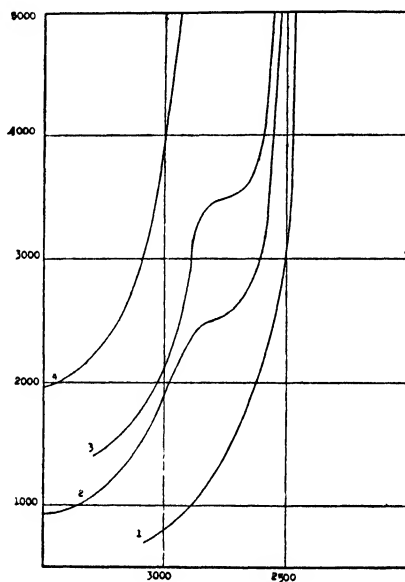


FIG. 1. Solutions radiated with x-rays. Curve 1, unirradiated (control), Curve 2, radiated 5 hours; Curve 3, radiated 14 hours; Curve 4, radiated 27 hours. Ordinates plotted as E , abscissæ as \AA .

sorption of ultra-violet light. The step which makes its appearance in the curve following short radiation is again flattened out with longer radiation.

Fig. 2 shows the change in the absorption curve of cholesterol following γ -ray radiation. The change is the same as with x-ray radiation.

It is interesting to note in this connection that Hess and Weinstock (1) were able to increase the ability of cholesterol to absorb

ultra-violet light by prolonged radiation with ultra-violet light much as we have done with short wave-length radiation.

We can conclude from these results that x-rays and γ -rays do cause a definite change in the molecule of cholesterol.

Chemical examination of the radiated solutions as compared with the unirradiated confirms the results obtained with the spectroscope.

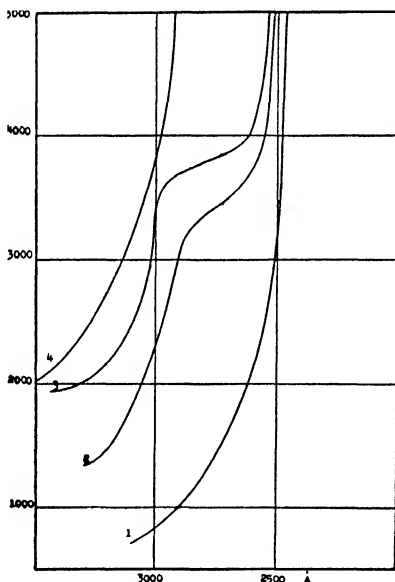


FIG. 2. Solutions radiated with γ -rays. Curve 1, unirradiated (control), Curve 2, radiated 5160 mch, Curve 3, radiated 16,950 mch; Curve 4, radiated 25,329 mch.

Fig. 3 shows the diminution of cholesterol with increasing amounts of x-ray radiation.

Fig. 4 shows a similar change brought about with γ -rays. 1 hour of x-ray causes approximately the same change as 1000 millicurie hours of γ -rays. These curves indicate that the solution is not approaching a condition of equilibrium and that doses greater than those which we have given would result in still greater changes.

A solution of cholesterol in chloroform which had been radiated

for 26 hours under the x-ray tube was allowed to evaporate to dryness. Instead of the customary white crystals a brown wax-like substance was obtained.

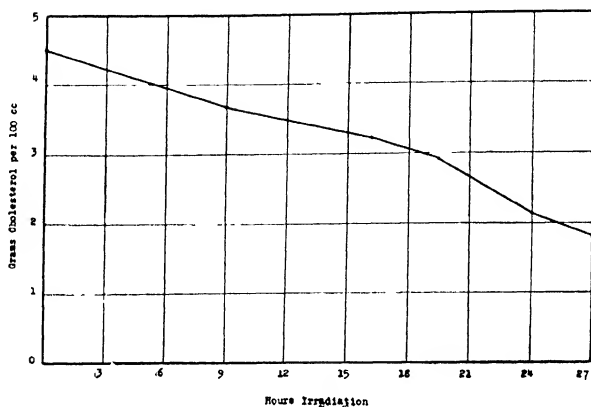


FIG. 3

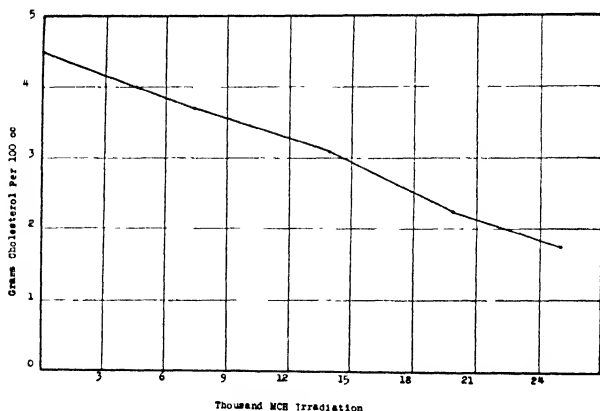


FIG. 4.

The readings taken with the polariscope show a slight but progressive change for the shorter doses but not for the longer periods of radiation. Table I shows this change.

Evidently that part of the molecule which is responsible for the optical activity is not appreciably affected by the radiation.

Description of Apparatus and Methods.

The spectroscope which was used in these experiments was a Hilger quartz spectrograph type C with a Hilger sector photometer. An under water spark served as the source of illumination. The curves are plotted wave-lengths in Å as abscissæ and extinction coefficients (E) as ordinates. The extinction coefficient (E) is defined.

$$E = \frac{1}{D} \times \frac{1}{C} \times \log I - \log I_1$$

where D = thickness of absorption cell in cm.

I = intensity of light incident on first surface of liquid

I_1 = intensity of light transmitted

C = concentration

TABLE I

γ -Ray radiation		x -Ray radiation	
<i>mch</i> *		<i>hrs</i>	
0	359 070°	0	359 058°
5100	359 240°	5	359 257°
12150	359 438°	14	359 410°
17000	359 095°	22	359 135°
25000	359 000°	26	Colored solution

* mch = millicurie hours

Rotation of wave-length $\lambda = 5460.7 \text{ Å}$

Cholesterol, 0.91 per cent in chloroform

Length of tube, 10 cm Temperature 20–24°.

The method of chemical examination is as follows. 1 cc. of the cholesterol solution is diluted to 10 cc. with chloroform, 2 cc. of acetic anhydride, and 0.1 cc. of concentrated H_2SO_4 . A standard solution containing 10 cc. of 0.08 mg. of cholesterol per cc. is treated in the same manner. Both are allowed to stand 30 minutes in a dark room at room temperature and then compared on a colorimeter.

CONCLUSIONS.

x -Ray and γ -ray radiation produce a definite chemical change in cholesterol dissolved in chloroform or absolute alcohol. That part

of the molecule which is responsible for the ultra-violet absorption and for the chemical reaction is changed more than the part which is optically active.

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ACID- AND BASE-FORMING ELEMENTS IN FOODS.

A CORRECTION.

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(Received for publication, March 16, 1927.)

In the report on the acid- and base-forming elements in foods¹ a large error has been discovered in the figure given for the nitrogen content of eggs. This figure (7767 mg.) represents the nitrogen content of the *dried* material and should be divided by 4.57, giving 1700 mg. of nitrogen per 100 gm. on the "as eaten" basis.

This mistake has also affected certain data on nitrogen in the "Studies in the Mineral Metabolism of Adult Man"² published by the University of California Press.

¹ Clark, G. W , *J Biol. Chem* , 1925, lxx, 597.

² Clark, G. W , *Univ Calif Pub Physiol.*, 1926, v, No 17, 195.

FACTORS INVOLVED IN THE REACTION CHANGES OF HUMAN SALIVA.*

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(Received for publication, February 28, 1927.)

The fact that deposits of calculus on the teeth frequently lead to irritation at the gingival margin has caused many to believe that these deposits and the subsequent irritation constitute the initial phase in the development of one form of pyorrhea. Although the relationship of the reaction of the saliva to deposition of calculus (tartar) on the teeth has been the object of many investigations, the exact chemical and physical processes involved in the formation and deposition of calculus are as yet unexplained. Marshall's (1) work on the "salivary index" was a study of the buffer value and did not give any idea of the true hydrogen ion concentration of the saliva. Using a hydrogen electrode, Foa (2) and Kirk (3) made the earliest attempts to determine the true reaction of saliva. It is obvious however, that the presence of carbon dioxide, ammonium salts, and proteins would make it difficult to determine accurately the initial reaction. The use of the open hydrogen electrode in such studies is therefore a questionable procedure. More recent work on the reaction of saliva is that of Starr (4) with observations on 610 specimens from 228 healthy normal subjects, that of Bloomfield and Huck (5) with 102 samples from 52 healthy subjects, and that of Bunzell (6) dealing with 274

* This work, carried out under the auspices of the original California Stomatological Research Group, was supported in part by grants from the Carnegie Corporation, the American Dental Association, and the Associated Radiographic Laboratories of San Francisco.

† The material submitted in this paper forms part of a thesis submitted by Kenneth L. Carter in partial fulfillment of the requirements for the degree of Master of Science in the Graduate School of the University of California.

subjects of varying ages. All of these investigators employed colorimetric methods. While Starr found variations from pH 5.75 to 7.05, 86 per cent were between pH 6.35 and 6.80. The median, mean, and mode¹ were at practically the same point, 6.60, showing that a sufficient number of subjects had been taken to approximate, very closely, a normal distribution. Bunzell separated his subjects according to sex and age. For 50 school boys he found an average pH of 6.64 and for 50 school girls an average of 6.62. For 50 female medical students the average was 6.49, for nine aged men (averaging 69 years) the average was 6.24, and for a group of aged women (averaging 74 years) the pH averaged 6.00. Bunzell's work indicates that the saliva becomes more acid with advancing age and that sex seems to have no bearing on the reaction.

Those who believe that caries is primarily an external process stress the solvent action of acids in the saliva. There is, however, little or no experimental proof involving pH determinations to support this idea. On the contrary, Lothrop and Gies (7), Pohle and Strebing (8), and Gans (9) find no correlation whatever between existing dental conditions and the reaction of an individual's saliva.

The work reported in this paper includes first, a comparative study of the reaction of saliva in the glands with that freshly expectorated; and second, a study of the changes in reaction resulting from the incubation of saliva, attempting thereby to simulate as far as is possible natural conditions for the action of bacteria and enzymes.

Methods.

A small hydrogen electrode was first considered but the idea that it was necessary to keep the saliva in a closed system in order to prevent loss of carbon dioxide, together with presence of ammonium salts and proteins, led us to adopt a colorimetric method. To accomplish the first part of the problem it was necessary to devise a single piece of apparatus which would serve both as a cannula and as the tube in which the reaction could be determined directly. The apparatus developed for this purpose is shown in

¹ Median, mean, and mode are used in a statistical sense.

Fig. 1, Parts I, II, and III. The cannula part is calibrated in tenths of a cc. from the tip of the cannula to 0.4 cc. The comparator, or barrel part, is calibrated in 0.1 cc. from the upper stop-cock to 1.4 cc. These figures were used because it was found that 0.4 cc. of indicator to 1.0 cc. of saliva or buffer gave the maximum color difference with these small diameter tubes. The test-tubes were cut from the same piece of Pyrex tubing as was the barrel of the cannula, so that the diameter, thickness of wall, and tint of glass

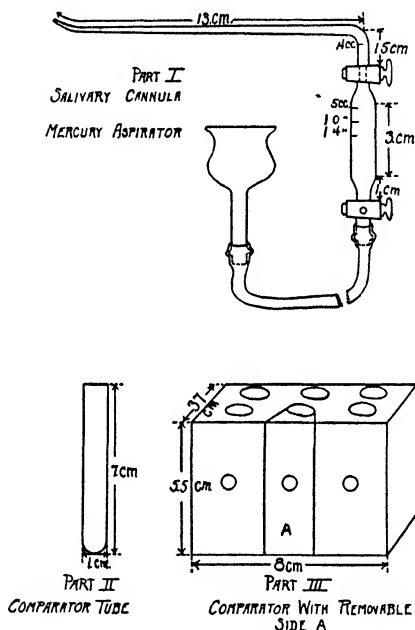


FIG. 1.

were exactly the same. The comparison was made in the block shown in Part III; the removable section A permitted the cannula to be placed directly in the block.

Buffer Standards.—The series of buffer standards were made from the phosphate mixtures of Sorensen (10) as described by Clark (11). Baker's c.p. salts were recrystallized. The values given by Clark were plotted as two curves and interpolation made to secure the proportions of the mono- and dibasic salts for a

series of buffers, ranging from 5.2 to 7.8, varying by 0.1 pH unit. These standards were kept in Pyrex flasks which had been coated inside with a mixture of equal parts of beeswax and paraffin. Prior to use they were checked against a hydrogen electrode.

Indicators.—Brom-thymol blue was the indicator most used since it covers the pH range between 6.0 and 7.8. For ranges from 5.2 to 6.8 brom-cresol purple was used. The 0.04 per cent solution of each indicator was prepared according to Clark's (11) directions.

Procedure for Determining the pH of Saliva.—The cannula-comparator was completely filled with mercury by raising the reservoir. After drawing in 0.4 cc. of indicator, the lower stop-cock was closed and the cannula inserted into the duct (or into the flask of incubated saliva). The mercury reservoir was then lowered a few cc. and the stop-cock partially opened, and saliva was drawn in until the mixture reached the 1.4 cc mark on the barrel. From 2 to 5 minutes of most careful manipulation were necessary to obtain 1 cc. of saliva by cannulation of one of the salivary glands. Both stop-cocks were then closed, the comparator gently shaken, and comparison made with the proper buffer standards. These standards were made up by adding 0.8 cc. of indicator to 2.0 cc. of buffer solution. A tube of saliva was placed behind each buffer tube and a tube of water behind the barrel of the cannula containing the saliva. All comparisons were made with a Palo Daylight lamp as background. The above apparatus was used in making all pH determinations because of its convenience and, more important, the small amount of saliva needed. It also obviated any changes in the carbon dioxide content which ordinary pipetting might introduce.

Although Michaelis and Pechstein (12) and Starr (4) showed that dilution of saliva has but little effect on pH, we preferred to eliminate this factor entirely and used only undiluted saliva.

Carbon Dioxide—The method of Van Slyke (13) was used.

EXPERIMENTAL.

In Table I data are presented showing the differences in reaction between the saliva as it is obtained directly from the glands and that freshly expectorated. If one considers the extensive changes

in the reaction of saliva produced by various types of stimulation of the salivary glands (14-16), the differences in pH (0.1) shown in Table I seem negligible. A comparison of the pH of quiescent and paraffin-activated saliva is shown in Table II. In these determinations a sample of quiescent saliva was taken and the pH determined. Meanwhile a piece of paraffin was placed in the mouth and after a very brief period of chewing, never exceeding 2 minutes, the reaction of this activated saliva was determined. As can be seen the rise in pH of the activated sample may be as

TABLE I
Comparison of the pH of Cannulated and Expectorated Saliva

Subject	Cannulated		Expectorated	Difference
	Parotid gland	Sublingual gland		
	pH	pH	pH	pH
K. C.		6 40	6 50	0 10
M. E.	6 40		6 52	0 12
K. C.		6 25	6 35	0 10

TABLE II
Comparison of pH of Resting and Paraffin-Activated Saliva

Subject	Resting	Paraffin-activated	Difference
	pH	pH	pH
G. C.	7 15	7 30	0 15
K. C.	5 95	6 80	0 85
H. U.	6 95	7 35	0 40
K. C.	6 10	7 10	1 00
L. L.	6 40	7 30	0 90

much as a whole unit. The slight difference between cannulated² and freshly expectorated samples, together with the difficulties encountered in cannulating the glands, made it undesirable to pursue further this part of the investigation.

Since it required about 40 minutes to collect adequate amounts of saliva it seemed necessary to determine the changes taking

² It should be pointed out that the cannulated samples were obtained under the influence of several types of stimuli (pressure, pain, psychic, etc.) and it is possible that the secretions of the different salivary glands are actually more acid than is indicated by our results.

place during that interval. A definite decrease in carbon dioxide was anticipated but the actual findings showed only a very slight loss, even when the saliva stood in an open container. Data, typical of the results obtained, are presented in Table III. While Findlay (17) found that carbon dioxide was more soluble in colloidal solutions, it does not seem that such an explanation could account for retention of the gas in saliva, considering that this fluid contains around 20 volume per cent, while 0.06 per cent (usually 0.04) is the highest value we can assign to this gas in laboratory air. Henderson and Stehle (18) found the carbon dioxide tension in the tissues of the mouth to be 54 mm. (58 mm. in saliva itself) therefore, when saliva is exposed to the air where the carbon dioxide is but 4×10^{-4} per cent of the total tension, one would expect appreciable loss of the gas. The matter became

TABLE III

Changes in Carbon Dioxide Content when Saliva is Kept in Open Container.

Subject K C, Nov 17, 1925

Time incubated	Carbon dioxide	pH
<i>hrs</i>	<i>vol per cent</i>	
At once.	27.4	7.10
$\frac{1}{2}$	26.5	7.15
1	26.0	7.30
$1\frac{1}{2}$	26.9	7.70

more clear when determinations were made on saliva which was kept closely stoppered; here, instead of a lower or a constant value, there was a definite increase. It was evident that carbon dioxide was being formed in the saliva and in the open vessels the gas formed was enough to replace that which was being lost, hence a nearly constant value was always found. It was also noticed that the formation of carbon dioxide was more vigorous in the thicker, heavier salivas than in the thin type. We were fortunate in securing a sample of very thick, sticky saliva from subject R. L. during an intense paroxysm of calculus deposition. The data presented in Table IV show the rapidity and extent of the carbon dioxide formation.

It was thought that the formation of carbon dioxide was too rapid to be attributed to bacterial action. In order to ascertain

more definitely the mechanism of this carbon dioxide formation it was necessary to select a substance which would be strongly germicidal for the ordinary microorganisms in the buccal cavity without inhibiting the action of any enzymes present. Accordingly, an excess of chloroform was added to half of a fresh sample

TABLE IV.

Changes in Carbon Dioxide Content When Saliva Is Kept in Closed Container.

Subject R. L., Nov. 18, 1925.

Time incubated	Carbon dioxide	pH
<i>min.</i>	<i>vol per cent</i>	
At once.	22 3	7 00
30	23 1	7 25
60	26 0	7 25

TABLE V

Chloroform Does Not Inhibit Carbon Dioxide Production in Saliva (Open Container)

Time incubated	Carbon dioxide	
	Chloroform-treated	Control
	<i>vol per cent</i>	<i>vol per cent</i>
At once	39 9	39 6
30 min	39 5	39 3
1 hr	39 9	39 9
2 hrs.	36 1*	36 4*

* As can be seen from the data presented in Tables I to V and Figs 2 to 4, the production of carbon dioxide is quite rapid from the time of collection up to approximately 2 hours, subsequently, from the 2nd to the 5th hours, there is usually a drop in the carbon dioxide content which in turn is followed by a steady increase in the content of the gas. These changes can be seen most readily by glancing at Figs 3 and 4. Had Hall and Westbay followed the changes in the carbon dioxide content in conjunction with their observations on the pH, they would no doubt have noted the changes mentioned above.

of saliva and, with the untreated portion as a control, the carbon dioxide changes followed through 2 hours of incubation. Since the control and the chloroform-treated samples show similar and concurrent variations, it is evident that chloroform did not inhibit the gas formation (see Table V). Had preservation been

effected there should have been a noticeable decrease in the carbon dioxide content of the samples containing chloroform. The rapidity of the increase in carbon dioxide and the failure of chloroform to check the gas formation suggest very strongly that bacterial action is not responsible for the immediate production of carbon dioxide in the saliva. Since enzymes are "poisoned"³ by most of the heavy metals, such as mercury, experiments were made to determine the effects of mercuric chloride on the gas formation. Mercuric chloride (1 cc. of a 1 per cent solution per 100 cc. of saliva) was added to one-half of a fresh sample of saliva and with the other portion as a control, changes in the carbon dioxide content were followed as was done with chloroform. The

TABLE VI.

Mercuric Chloride Inhibits Production of Carbon Dioxide in Saliva (Open Container)

Subject	Time incubated	Carbon dioxide	
		Mercuric chloride-treated	Control
	hrs	vol per cent	vol per cent
L. L.	At once	14.5	14.5
"	1	10.7	13.5
"	2	9.8	15.5
K. C.	At once.	27.8	28.3
"	1	25.4	28.4
"	2	26.2	28.5

results, given in Table VI, show that gas formation was inhibited by the mercuric chloride. The results presented in Tables III, IV, and VI suggest very strongly that the production of carbon dioxide is due principally to enzymatic action.

³ Since the composition of enzymes is unknown it is impossible to explain the so called poisoning by mercury. As could be expected, we found considerable material precipitated whenever mercuric chloride was added to saliva. After removal of this precipitate by centrifuging, the supernatant fluid gave a black precipitate with hydrogen sulfide, indicating that we had added an excess of the reagent. These heavy metal-protein complexes are appreciably soluble in physiological salt solution (0.9 per cent NaCl) and in this condition are relatively unstable. We are, therefore, unable to exclude decomposition of the Hg-protein complex by the hydrogen sulfide.

As shown in Tables III and IV a rise in pH generally occurred simultaneously with increases in the carbon dioxide content of incubated saliva. Hall and Westbay (19) observed similar changes in about half of their samples and suggest loss of carbon dioxide as the possible cause. However, as we have pointed out in the preceding pages, it is not possible to correlate the changes in pH with changes in the carbon dioxide content. This anomalous condition shows that other agents are increasing the pH more effectively than carbon dioxide can decrease it. Although considerable ammonia is present in saliva (20-23), the extent to which it might form during incubation was not known and it occurred to us that production of this compound might partially or wholly

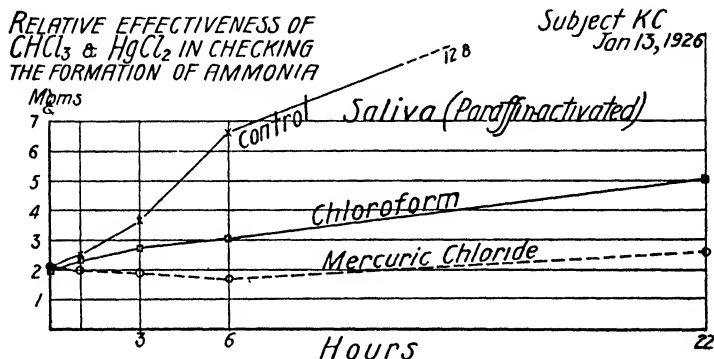


FIG. 2.

account for the peculiar changes in pH. If the ammonia content of the saliva were due to bacterial action upon the urea present, as stated by Schmitz (20), the formation should be inhibited more or less completely by chloroform. The diverse action of chloroform and mercuric chloride upon carbon dioxide production in saliva led us to repeat the experiments with these preservatives to determine their action upon the formation of ammonia. For this purpose a composite sample of paraffin-activated saliva was divided into three portions; to one was added an excess of chloroform, to the second the customary amount of mercuric chloride, while the third portion was retained, untreated, for control. The results, given in Fig. 2, show that there was a very marked and rapid increase in the ammonia content of the control sample, over

300 per cent in 6 hours. Chloroform partially inhibited the formation of ammonia, an increase of only 44 per cent in 6 hours in contrast to the complete failure in inhibiting the formation of

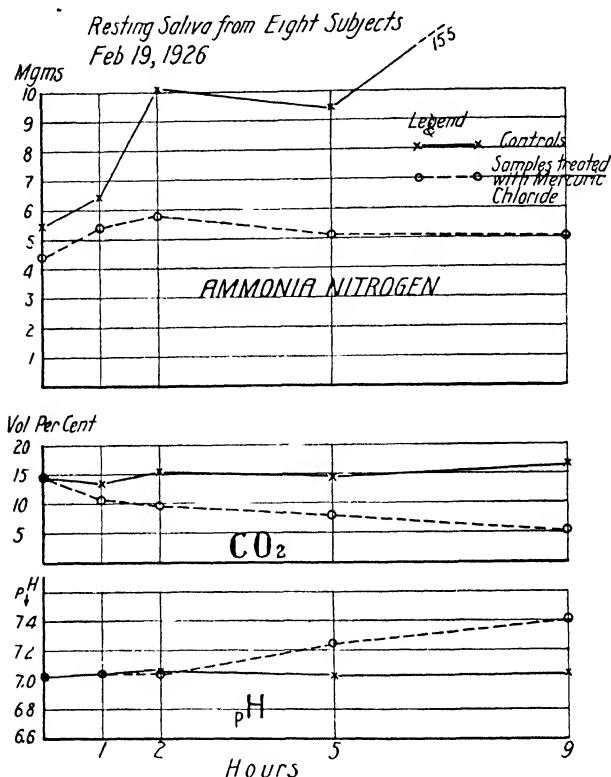


FIG. 3.

carbon dioxide. On the other hand, mercuric chloride was as effective in inhibiting ammonia production,⁴ as was observed in

⁴ A number of experiments was conducted to check the ammonia values obtained by permutit absorption with those obtained by aeration. The results showed that the more rapid permutit method is dependable and that it may be used safely with complex fluids such as saliva. Experiments with solutions of ammonium sulfate and saliva also showed that the amount of mercuric chloride used had no effect either upon the absorption by the permutit or subsequently on Nesslerization. Under existing conditions

the experiments in carbon dioxide formation. The results with chloroform are contrary to those of Updegraff and Lewis (23) and place some doubt on the view expressed by Schmitz (20) that the total ammonia in the saliva is the result of bacterial action

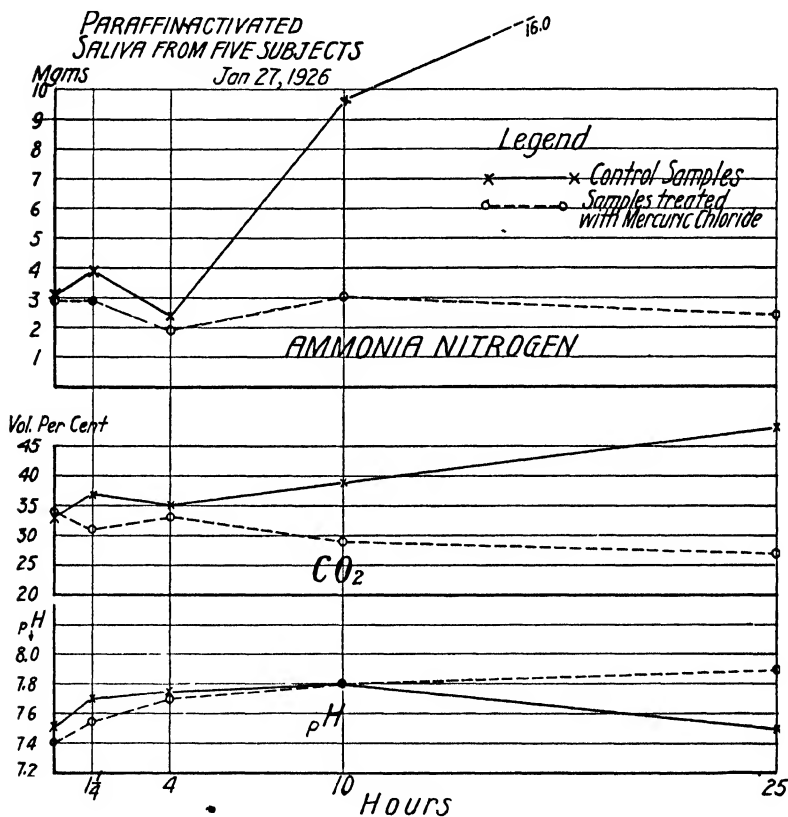


FIG. 4

upon the urea. Having found definite increases in the ammonia content of incubated saliva, the next experiments included simultaneous determinations of ammonia, carbon dioxide, and pH.

it is also unlikely that complex ammonium-mercury compounds were formed, certainly not in amounts greater than the inherent analytical errors.

Saliva was collected from several subjects (either resting or paraffin-activated) and, after mixing thoroughly, the composite divided into two equal portions, mercuric chloride was added to one-half and the other portion kept as a control. The results, given in Figs 3 and 4, show the extent of the concurrent variations in the pH, carbon dioxide, and ammonia. Considering first the samples treated with mercuric chloride, it can be seen that, with an almost constant value for ammonia, there has been a gradual rise in pH which is apparently associated with a progressive loss of carbon dioxide. However, in the corresponding untreated samples both the ammonia and carbon dioxide increased several per cent without any significant changes in pH. (Considering the changes at the end of 9 and 10 hours, CO_2 increased 1.8 volume per cent, pH increased 0.05 unit in resting saliva. In paraffin-activated saliva, CO_2 increased 6.3 per cent, ammonia nitrogen increased 7.8 mg, pH increased 0.30.)

The results of a large number of analyses⁵ show that resting saliva contains from 20 to 30 per cent as much carbon dioxide (as bicarbonate) and from 3 to 6 times as much inorganic phosphate as does blood plasma. Accepting an average pH of 6.6 (4-6) (9), it is evident that saliva is a well buffered mixture and would be able to neutralize large amounts of hydrogen or hydroxyl ions without showing appreciable changes in the pH. In this connection it should be noted that Bloomfield and Huck (5) gave human subjects as much as 20 gm. of sodium bicarbonate per day without effecting any changes in the pH of the saliva.

If deposits of calculus are associated with changes in reaction, it would seem that definite increases in the pH should cause measurable decreases in the amount of calcium held in solution. A number of calcium determinations made in conjunction with the data presented in Figs. 3 and 4 exhibited no such relationship. Thus in one instance the determinations made 12 hours apart showed an increase in the carbon dioxide of 9.2 volumes per cent, a decrease in the pH of 0.3 unit, with a 30 per cent decrease in the calcium content. When the pH reaches 8.0 or more, it is quite evident that much of the calcium would be precipitated (Ca mucinate, $\text{Ca}_3(\text{PO}_4)_2$). Although the pH may subsequently de-

⁵ These figures were obtained from a large number of analyses of saliva (24, 25).

crease by a whole unit or more, the concentration of hydrogen ions is never sufficient to redissolve the precipitate. This explanation would also apply to the results reported by Hall and Westbay (19).

SUMMARY.

The results of the experiments reported in this paper indicate that:

1. The saliva obtained by direct cannulation of the human parotid and sublingual glands is but slightly more acid, 0.1 pH, than freshly expectorated resting saliva

2. Although the volume per cent of carbon dioxide is much higher in paraffin-activated saliva, it varies in much the same manner as in resting saliva.

3. Samples of either resting or paraffin-activated saliva may stand for several hours without showing appreciable changes in the carbon dioxide content. This is explained by an equilibrium between the carbon dioxide escaping and that being formed.

4. The formation of carbon dioxide is probably the result of enzymatic action.

5. There is apparently some ammonia formed by bacterial action. It is thought, however, that most of it is the result of enzymatic action

6. There is no demonstrable relationship between the pH, the volume per cent of carbon dioxide, and the ammonia content. The pH changes in saliva apparently involve other constituents than those studied. To complete this aspect of the work properly it will be necessary to make complete analyses of a large number of samples of saliva.

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NUTRIENT IONS OF PLANTS AND THE ION ACTIVATION OF PLANT ENZYMES.*

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INTRODUCTION.

The rôle of salts and of their ions in the metabolism of the cell still remains one of the most important problems in the biological sciences.

It was believed that enzyme activity would be modified in plants when growing in solutions lacking certain salts or ions, and to test this idea out a number of experiments was undertaken with sugar beets as the test crop. At certain arbitrary periods during the growth of the plants, leaf material was gathered and the enzymes extracted. Certain tests were then made on these enzymes to determine their activity under various conditions.

Method.

1. Sand Cultures.

The plants used in the experiments were grown in pure quartz sand cultures in enamelled earthenware crocks of 2 gallon capacity. There were seventeen of these cultures, nine being reserved to be watered with a complete nutrient solution (containing all the essential salts in proper balance) and the other eight to be watered with a solution like the above with the exception of a deficiency of potassium. In this solution another salt, sodium, was substituted for the potassium. Care had to be exercised also

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to adjust the solution to the same osmotic concentration as that of the complete solution. The construction of the cultures is shown in Fig. 1. The gravel layer at the top of the crock, as shown in the sketch acted as a mulch to check a too rapid evaporation of water and to restrain the growth of algæ.

The sugar beet plants¹ were grown in the field from pure line seed. When about 2 months old they were carefully transplanted to pots and brought into the greenhouse. Here they were left for a while to become accustomed to the new environmental conditions before being again transplanted into the culture crocks

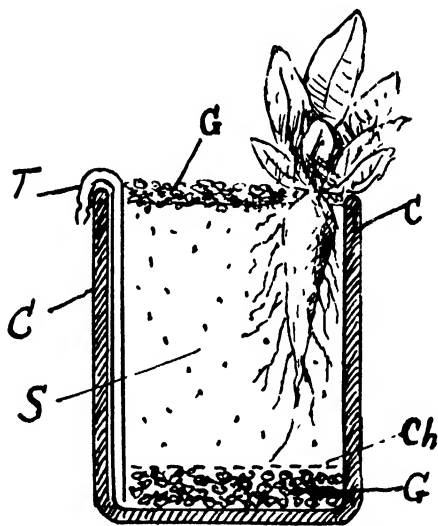


FIG 1 Details of quartz sand cultures C, crock, T, glass tube for aeration and exchange of solution; G, quartz gravel, S, quartz sand; Ch, cloth

described above. Transplanting was done carefully and the roots freed of soil particles by a jet of water. Three plants of uniform size were put in each crock.

The complete nutrient solution developed by Stoklasa and Matousek (11) and reported as supporting luxuriant growth was slightly modified for our purpose and also found to give excellent

¹ We are indebted to E E Down, Plant Breeder, for these excellent plants.

growth. A glance at Table I will show that the modification consisted in eliminating certain ions that occurred frequently and in using magnesium sulfate in the place of calcium sulfate.

The two sets of sand cultures thus prepared were saturated with their respective solutions, which in 10 minutes were partially withdrawn, by means of a suction pump, to a final water content of 16 per cent. The total weight was then recorded on the jar. At weekly intervals the solutions were renewed in the same way and then sucked out to original weight. By this means the water content from week to week throughout the growing season was approximately maintained. The cultures were set on a rotating table in the greenhouse. Additional light (five 200 watt nitrogen-filled Mazda lamps) was used during the winter months. The plants were carefully watched and kept free from insect and

TABLE I.
*Salts and Volume Molecular Concentration for Three Types of
Solutions Compared*

	KNO ₃	CaH ₂ PO ₄	MgSO ₄	NaCl	FeCl ₃	CaSO ₄	MgCl ₂	NaNO ₃
	gm	gm	gm	gm	gm	gm	gm	gm
Complete	1 150 0	906 0	246 0	100 0	0 10			
Minus potassium		0 906 0	246 0	100 0	0 101			0 96
Stoklasa and Matousek	1 150 0	500		0 100 0	0 101 0	200 0	100	

fungous pests. The tests on these plants were made at two different periods during their growth, February 10 to 14 and April 7 to 13. On the first date, about 2½ months after being transplanted, the plants were in good vigorous condition, those in the complete nutrient being from one-third to one-fourth larger than the plants in the potassium-deficient nutrient. At the beginning of the second period the plants in the potassium-deficient nutrient had gained on an average of 46 per cent on their previous measurements, while those in the complete nutrient had made a gain of 75 per cent. Since all other conditions for growth were the same, it is evident that potassium has much to do with the vigor and growth of plants. What then can be the influence of a potassium-deficient nutrient on enzyme activity? How would the enzymes extracted from the leaves of plants in these two types of nutrient differ in amount, character, and salt activation?

2. Enzyme Preparations and Preliminary Tests.

The two enzymes for study were those chiefly responsible for carbohydrate changes, amylase and saccharase. Both of these were extracted from the leaves at the same time by the following method: 20 to 30 gm. of fresh leaf material were shredded up after the petioles had been thrown out. A definite weighed sample was then mixed with clean quartz sand and ground in a porcelain mortar until it had become a thin paste. The juice was then pressed out by hand through cheese-cloth, diluted with an equal quantity of distilled water, and centrifuged for 5 minutes at 2000 R P.M. The clear liquid was pipetted off and used immediately. Toluene was added as a preservative where necessary. This method of making the enzyme preparation is preferable to that in which the leaf material is first dried and then extracted. The enzyme appears to be obtained in unmodified form by this method.

The presence of amylase in the sugar beet was demonstrated long ago by Gonnermann (8) and later by Brasse (2). No quantitative experiments were reported. Later Palladin and Popoff (9) isolated amylase from sugar beet leaves and the leaves of many other kinds of plants. These men reported in addition more amylase in young leaves than in older ones.

Saccharase was shown by Gonnermann (8) to be present in the sugar beet plant. Later Stoklasa, Jelínek and Vítex (10) found this enzyme in roots undergoing anaerobic respiration. Colin (4) made a study of the roots and leaves separately and found it always in the leaf, but absent in the roots that were growing normally. At the same time Bodnár (1) showed that it was absent in healthy roots but present in roots suffering from a disease, commonly known as "root-rot" (Schwanzfaule).

Several preliminary experiments, which will be briefly noted, had to be conducted in order to become acquainted with the activity of the enzyme preparation and the various experimental conditions. For these studies good vigorous plants were selected from the general supply brought into the greenhouse from the field and potted in their own soil. The enzyme preparations were so active that considerable dilution (10:1000) was necessary at the start. No buffers were used as it was thought that natural

ones in sufficient quantities were present in plant juices. Furthermore, under the conditions arbitrarily set, it was necessary to study the influence of alkali salts without the disturbance of other buffers. The salts used in the study of enzyme activation were potassium nitrate, sodium nitrate, and potassium chloride. Studies were also made to determine the proper salt concentrations and to find out which salt and which ion exerted the greatest influence.

In testing for amylase and saccharase in young and older leaves it was found that young leaves showed more amylase than older ones. It did not appear to be a matter of the size, but just a matter of age. In regard to saccharase activity the young leaves contained more enzyme than the older ones. Great care then had to be exercised in the collection of material for comparative tests. All leaf collections were made at noon.

The method of procedure for determining the diastatic power or the sucroclastic activity of the leaf juice was that used by the senior author in his studies on the enzymes of the potato leaf (5, 6). For the amylase studies the method consisted in incubating a 5 cc. sample for 24 hours at 38°C., and then determining by Wohlgemuth's (13) colorimetric iodine method the diastatic power. In the tables this is represented as $D_{24h}^{38^\circ}$ (diastatic power at 38°C. for 24 hours). The 5 cc. sample contains a definite amount of a 1 per cent starch solution, a definite concentration of enzymatic juice, and 0.1 cc. of toluene as a preservative.

The details of the method as used in the studies on saccharase are as follows: 2 cc. of enzymatic preparation are put into a total of 50 cc. of a solution which is made up of saccharase at 5 per cent, toluene (0.1 cc.) as a preservative, and the necessary amount of activating salt to reach the concentration called for in the test. This solution is then put into the incubator at 38°C. At certain time intervals, previously determined upon, 5 cc. quantities are withdrawn and treated with 1.5 cc. of lead subacetate (saturated solution diluted 2:5). After shaking, 0.5 cc. of a 12.8 per cent Na_2SO_4 solution is introduced and the solution shaken again. It is now centrifuged and 4 cc. of the clear liquid are mixed with 1 cc. of a 5 per cent solution of Na_2CO_3 (made from crystals). This is again centrifuged and the clear solution is polarized in a 20 cm. tube at 20°C. The constant (k) of the

reaction is calculated according to von Euler and Svanberg (7),
 $k = \frac{1}{t} + \log \frac{R + L}{d + L}$ where t is time in hours from the beginning
of the reaction, R the initial rotation, L the final rotation, and
 d the observed rotation at t time.

TABLE II
Amylase Activity in Plants under Two Types of Nutrient Conditions.

Type of nutrition	$D_{24h}^{38^\circ}$		$\frac{D \text{ Apr}}{D \text{ Feb}}$
	Feb 10	Apr 8	
Complete	18	5	0 28
Minus potassium	33	25	0 75

TABLE III
Salt Activation of Amylase Extracted from Leaves of Plants Growing in Cultures Containing a Complete Nutrient as Compared with Similar Samples of Amylase from Leaves of Plants Growing in Cultures Otherwise Complete with the Exception of Potassium

Concentration of salt	1st period, Feb 10, 1925		2nd period, Apr 8, 1925	
	Complete	Minus potassium	Complete	Minus potassium
	per cent	per cent	per cent	per cent
Check.	100	100	100	100
NKNO ₃ .	139	300	294	588
" NaNO ₃	139	300	147	588
" KCl	278	600	789	1176
Check.	100	100	100	100
0.01 NKNO ₃	129	200	100	400
0.01 "NaNO ₃	118	200	100	400
0.01 "KCl	294	400	100	1600

3. Experiments on Enzyme Preparations Obtained from Plants Growing under Two Sets of Nutrient Conditions.

With these preliminary experiments out of the way, it was next in order to extract the juice from the leaves of plants growing under conditions of complete nutrition and also under conditions of a nutrient lacking potassium, and make certain comparative

studies of the enzyme preparation thus obtained. The tests were made as noted above at two different periods during the growth of the plants, the first in February about 2½ months after the cultures had been set up and the second in April. The same

TABLE IV

Values of k for Saccharase in Plants Growing in Cultures Treated with a Complete Nutrient Solution and with Solution Lacking Potassium.

Type of nutrition	$k \cdot 10^4$		$\frac{k \text{ Apr}}{k \text{ Feb}}$
	Started Feb 14	Started Apr 10	
Complete	8 3	29 3	3 5
Minus potassium	27 5	50 7	1 8

TABLE V

Values of k for Saccharase Obtained from Plants Growing under Complete and Minus Potassium Nutrient Conditions for the Two Experimental Periods.

Tests made in time periods varying from 2 days to 32 days

Experimental period	Complete				Minus potassium			
	<i>t</i> hrs	α	<i>k</i> 10 ⁴	<i>k</i>	<i>t</i> hrs	α	<i>k</i> 10 ⁴	<i>k</i>
<i>1925</i>				<i>per cent</i>				<i>per cent</i>
Feb 10-14		+6 75				+6 68		
	46 1	+5 99	8 3	100	44	+4 46	27 5	100
	89 5	+5 40	7 8	95	95 2	+2 76	26 3	96
	256 8	+4 50	4 8	59	254 6	+0 72	18 7	68
	334 5	+4 29	4 1	50	325 5	+0 21	17 1	62
	546 3	+3 65	3 3	40	540 5	-0 78	14 4	52
	∞	-2 29			∞	-2 27		
Apr 8-13		+6 72				+6 78		
	40 2	+4 58	29 3	100	43 2	+3 18	50 7	100
	100 9	+2 36	28 5	97	84 8	+0 12	67 6	133
	385 8	-1.62	29 4	100	183 7	+1 68	63 1	125
	766 7	-1.80	16 6	57	277 8	-1 73	43 0	85
	∞	-2 28			756 7	-1 80	33 2	65
					∞	-2 31		

general outline of procedure and the same methods were followed as indicated in the preliminary studies.

The results of the study as far as amylase is concerned can be found in Tables II and III. It is first evident, Table II, that

amylase from leaves of plants growing in a complete nutrient solution is less in amount than amylase extracted from leaves of plants growing in a nutrient lacking potassium. This is true for both growth periods. In the earlier period the difference was approximately twice as much, while in the second period it was 5 times as much. Again it is evident that enzymatic activity decreases with age, the decrease being more marked in plants growing under complete nutrient conditions. In Table III the D (diastatic power) values in percentage are recorded and show that activation occurs with all salts used and to a much greater extent

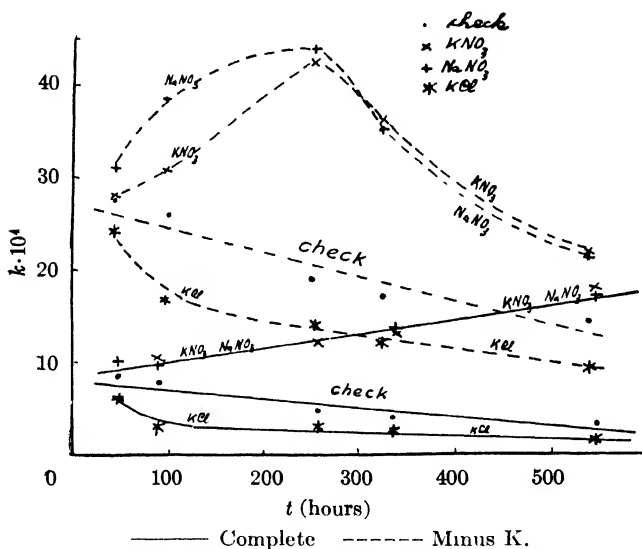


FIG 2 First experimental period, absolute values of K

in the material derived from plants growing in a potassium-deficient nutrient. The figures are more striking in the second period when the concentration of salts is 0.01 N.

The results of the study on saccharase are to be found in Tables IV and V. In the first of these two tables the k values for the enzyme are lower when obtained from plants growing under complete nutrient conditions than when taken from plants growing in potassium-deficient cultures. The difference is 2 or 3 times as much. It is also clear that the k values increased in the second period. With material from plants in the complete nu-

trient the increase was nearly 4 times, while in the other case it was nearly twice that of the first period.

In Table V a further difference is noted and that is in the behavior of k , or, for example, the course of the reaction. In the first period (February) k (percentage) decreased in both types of nutrition, while in the second period it was constant for 16 days

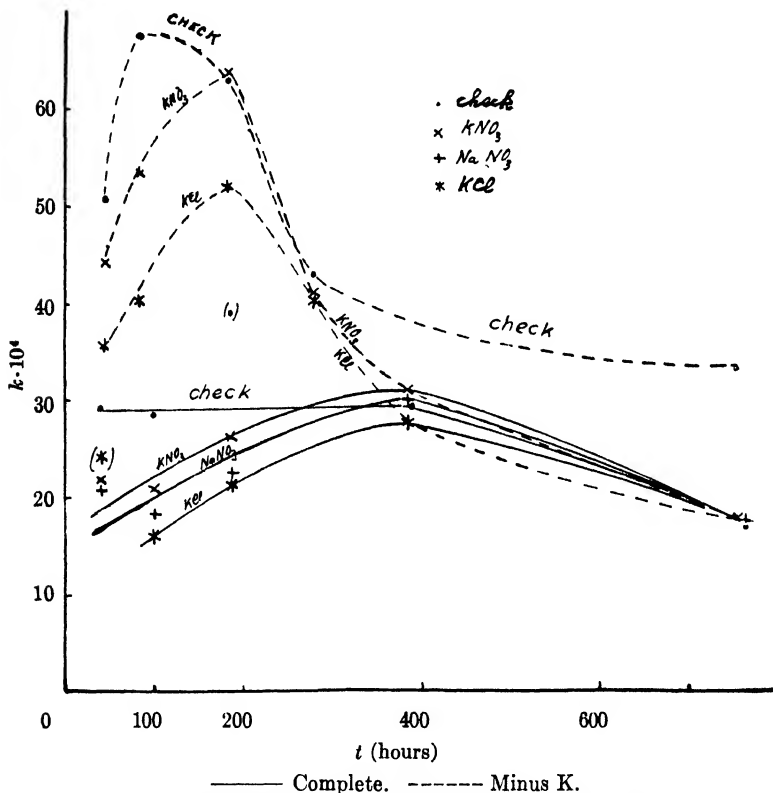


FIG. 3. Second experimental period, absolute values of K .

for the complete nutrient and increased to the 4th day in the minus potassium nutrient. This is shown more clearly in Figs. 2 and 3 when the curves marked "check" are observed. In these graphs the absolute values of k are plotted, however.

The influence of the different salts on the absolute values of k for the different types of nutrient conditions is also shown in

the above graphs, the use of which eliminates the necessity of publishing the many tables prepared. Nitrates, it is observed, with but one exception were activating while the chloride was inhibiting. The exception was for the salt NaNO_3 and for the material obtained from plants in the potassium-deficient nutrient and for the second period. In Fig 3, therefore, the curve for NaNO_3 is left out. It was also demonstrated in these experiments that there was a difference, through the use of salts, in the degree of enzyme activity between the two periods. There was twice as much in the earlier period as in the second.

Finally, as can be learned from Figs 2 and 3, the k curve rises or falls much more slowly when the enzyme preparation is obtained from plants in complete nutrient condition than in the enzyme preparation from plants in potassium-deficient cultures. This is true in both growth periods, but is especially marked in the second period.

In the first period, nitrates showed considerable activating power when the enzyme preparation was obtained from plants in the potassium-deficient nutrient. In other words the curves rise to a high maximum. In the second period the same was true, but to a greater degree.

DISCUSSION OF RESULTS

These experiments with amylase and saccharase go to show that ions may have a specific action and also an antagonistic one.

The type of nutrition and the physiological balance in a nutrient solution markedly influence the activation of enzymes by salts. Amylase preparations, for example, from leaves of plants in a complete nutrient, are not nearly as strongly activated as are the amylase preparations from leaves of plants growing in a potassium-deficient nutrient. In the latter case an unbalanced nutrient has induced an unbalanced salt or ion condition or perhaps a deficiency of a particular salt or ion in the plant and so affects the enzyme. When certain even weak concentrations, then, of these deficient salts or ions are used as activating agents the enzyme shows great activity. In the above experiments, since potassium was lacking, the plant was unable to obtain the chloride ion in sufficient quantity so that potassium chloride exerted a great influence as an activating agent.

These experiments show another thing of great importance. These enzymes occur in greater quantities in material from plants in potassium-deficient nutrient than in material from plants in complete nutrient. Brown and Morris (3) report the same result with leaves kept in darkness when compared with those in light. They conclude that this is a symptom of starvation. Our experiments show, however, (Table II) that with amylase the decrease in the amount as the plants age is less rapid in the potassium-deficient nutrient than in the complete one. In the case of saccharase (Table IV) the increase in amount as the plants age is also less rapid in the potassium-deficient nutrient. Sjoberg (12) noticed the decrease in the amount of amylase with age in certain tree leaves, but concluded that it was not due to external conditions. The experiments reported here show at least for sugar beets that external conditions are important.

SUMMARY.

This study of the rôle of electrolytes in regulating amylase and saccharase activity in the sugar beet under different nutrient conditions leads to the following conclusions.

1. Young leaves contain more of the enzyme than the older ones.

2. The quantity of the enzyme is controlled by the type of nutrition, being greater in plants growing in a potassium-deficient solution than in a complete nutrient solution.

3. Amylase is strongly activated by the chloride ion and weakly activated by the potassium, nitrate, and fluoride ions. Saccharase is activated by the nitrate ion and somewhat by the potassium ion, but paralyzed by the chloride ion. These two enzymes in turn seem to be differently activated depending on the type of nutrition

4. These actions are characteristically modified by the condition of potassium deficiency. Contrary to expectation the strongest activation is not with potassium. Potassium only slightly activates the enzymes which are merely activated by specific ions. This phenomenon is explained by supposing a differential absorption of nutrient ions from the solution and a specific action of the ions on the different enzymes.

5. In all enzymatic studies, the type or kind of nutrition is as important as a determination of pH values, temperature, etc.

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INTERMEDIARY CARBOHYDRATE METABOLISM.

II. KETOSIS IN PHLORHIZIN DIABETES.

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INTRODUCTION.

The object of this and the following paper, including the results of the calorimeter work (1), is to prove that a completely phlorhizinized dog is able to oxidize glucose as soon as the rate of its accumulation in the body exceeds the rate of its elimination by the kidneys. In order to show that glucose may be oxidized in such conditions after it is ingested, it is necessary to prove (1) an increase in the blood sugar; (2) a parallel protein-sparing action (nitrogen-sparing action); (3) simultaneous disappearance of ketosis (ketolytic action); (4) irreversible disappearance of some glucose in this process; and (5) a corresponding rise in respiratory quotient. If they all appear at once these are the most important criteria of the oxidation of glucose in the living animal organism.

Method.

1 gm. of Merck's phlorhizin, recrystallized, ground in oil was injected every day subcutaneously into female dogs which were healthy and had fasted for 2 days before the onset of phlorhizination. The glucose-feeding experiment was usually made between the 6th and 8th day of phlorhizination. In one instance it was made on the 3rd, while in another case it was performed on the 11th day of phlorhizination. All the dogs were showing at this time constant D:N ratio and high ketosis. After a good basal period was obtained 10.00 to 31.41 gm. of glucose dissolved in water were given to these dogs by stomach tube. The glucose was

c.p. Corn Products Refining Company or Eimer and Amend anhydrous. For every single experiment the reducing value of administered glucose was determined and corresponding correction on the weight of the glucose taken introduced. After the feeding of glucose the dogs were carefully watched either while lying on the board or held in the arms of the observer in order to control vomiting. If the dog vomited the experiment was discarded. After glucose ingestion samples of urine exactly corresponding to the given periods were collected quantitatively at 3 hour intervals over a certain time and nitrogen (macro-Kjeldahl method), sugar (Bertrand's technique), and total ketone bodies (Van Slyke's procedure) determined in each sample. Also as a routine procedure qualitative examination of the urine for albumin and for separate ketone bodies was made. Special care was taken to examine urine for these constituents as soon as it was obtained from the bladder, during both the day and the night. If there was any delay,¹ the urine was shaken with toluene and put in the ice box, covered with a layer of toluene. Bertrand's method applied to pure sugar solutions gave values which agreed within 1 per cent with the polariscopic determination of the same sugar solution (2). The interval of 3 hours between the collection of samples was long enough to determine the reducing power of the urine, digest it, and make the preliminary glucose precipitation for the determination of total ketone bodies. At important points glucose was determined in triplicate and quadruplicate. Pains were taken to obtain at the end of the experiment conditions closely approaching the initial ones as to the content of different constituents in the urine. Water was given to these dogs at equal intervals, either placed before them in a dish or introduced by stomach tube, in order to prevent retention of urine. The entire work has been done by the author.

Results.

Ten complete experiments with sugar feeding and one with fat feeding are given here (Tables I to IX, XII).

¹ Only when calorimeter experiments were made or blood samples drawn at the same time.

10 42 p m - 1 45 a m	1 091	0 358	4 28	1 40	3 92	0 82	0 27	6 05	0 109	0 036	0 053 2 42 a.m	0 030	44
Feb. 4													
1 45 a.m.- 5 42 a m.	1 572	0 398	5 76	1 41	3 66	0 78	0 26	5 75	0 489	0 124			
5 42 " - 8 42 "	1 338	0 446	4 44	1 48	3 32	0 20	0 07	1 48	0 579	0 193			39
8 42 a m.-11 16 a.m.	6 095	32 88			13 56		=	100 00					8 42 a.m.
					3 15								

Calculation on the basis of D:N 3 17 gives 13.56 gm of sugar recovery out of 15.00, which is 90.4 per cent.

TABLE III.
Experiment 4.

Dog 5 5th to 6th phlorhizin day Weight 10.6 kilos

Date and time	Urine									
	Nitrogen		Sugar		D N ratio	Extra sugar		Acetone bodies		Per hr
	Total	Per hr	Total	Per hr		Total	per cent	Total	gm	
	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
1925										
May 13										
10 26 a.m. - 1 38 p.m.	0 802	0 251	2 48	0 77	3 09			0 322	0 101	
1 38 p.m. - 4 53 "	1 277	0 393	4 32	1 33	3 39			0 681	0 210	
4 53 " - 9 50 "	1 560	0 315	5 60	1 13	3 59			0 764	0 154	
9 50 " - 1 38 a.m.	1 268	0 334	4 31	1 13	3 40			0 767	0 202	
1 41 a.m.	19 34 gm of glucose by stomach tube									
May 14										
1 38 a.m. - 4 57 a.m.	1 075	0 324	9 50	2 87	8 84	5 84	39 91	0 592	0 124	
4 57 " - 7 50 "	0 762	0 264	8 81	3 06	11 57	6 22	42 51	0 057	0 020	
7 50 " - 10 46 "	0 631	0 222	4 00	1 36	6 15	1 79	12 24	0 017	0 006	
10 46 " - 1 27 p.m.	0 649	0 242	2 69	1 00	4 14	0 48	3 28	0 062	0 023	
1 27 p.m. - 4 39 "	0 851	0 261	3 19	0 98	3 75	0 30	2 06	0 229	0 070	
						14 63	= 100 00			
4 39 p.m. - 7 41 p.m.	0 968	0 319	3 19	1 05	3 29	= 3 14		0 354	0 119	
7 41 " - 10 44 "	0 881	0 289	2 84	0 93	3 23			0 325	0 106	
May 14-15										
10 44 p.m. - 10 18 a.m.	3 612	0 312	11 14	0 96	3 08			1 842	0 159	

Recovery of extra sugar 14 63 gm of 19 34 gm given = 75.7 per cent on basis of D:N = 3.40

BLE
Experiment 5.

Dog 1. 4th to 5th phlorhizin day (second phlorhizinization) Weight Apr 26, 9.3 kilos

Date and time.	Urine									
	Nitrogen		Sugar		D N ratio	Extra sugar		Acetone bodies		Per hr gm
	Total	Per hr	Total	Per hr		Total	per cent	Total	gm	
	gm	gm	gm	gm		gm		gm	gm	
1925										
Apr. 26-27										
8 21 p.m - 6 08 p.m	7 219	0 331	28 29	1 30	3 92			4 603	0 211	
6 11 p.m.										
1934 gm of glucose by stomach tube										
6 08 p.m. - 9 08 p.m	0 872	0 291	12 38	4 13	14 20	8 98	57 68	0 207	0 069	
9 08 " - 12 08 a.m	0 781	0 260	7 20	2 40	9 22	4 15	26 65	0 081	0 027	
Apr 28										
12 08 a.m - 3 08 a.m	0 732	0 244	4 75	1 58	6 49	1 89	12 14	0 150	0 050	
3 08 " - 6 08 "	0 897	0 296	4 05	1 35	4 52	0 55	3 53	0 449	0 150	
						15 57	100 00			
6 08 a.m - 9 08 a.m	0 910	0 303	3 08	1 03	3 39			0 642	0 214	
9 08 " - 12 08 p.m	0 766	0 255	2 80	0 93	3 65			0 651	0 217	
12 08 p.m - 3 08 "	0 871	0 284	3 63	1 20	4 23			0 733	0 244	
3 08 " - 6 08 "	0 871	0 290	3 40	1 13	3 90			0 778	0 259	
Apr. 28-29										
6 08 p.m - 3 56 p.m	7 543	0 346								

Recovery of extra sugar 15 57 gm = 80.5 per cent on the basis of D·N = 3 90.

TABLE VI.
Experiment 7.

Dog 4. (Second phlorhizinization.) 7th to 9th phlorhizin day Weight 8.4 kilos.

Date and time	Urine								
	Nitrogen		Sugar		D N ratio	Extra sugar		Acetone bodies.	
	Total	Per hr	Total	Per hr		Total	Per hr.		
	gm	gm	gm	gm	gm	per cent	gm	gm.	
1925 May 27 5 37 a m.- 8 56 a.m.	1 047	0 316	3 73	1 13	3 56		0 856	0 258	
8 59 a.m.	29 00 gm of glucose by stomach tube								
8 56 a.m.-12 06 p.m.	1 180	0 373	8 59	2 71	7 29	4 39	20 36	0 575	0 182
12 06 p m - 2 56 "	0 838	0 296	9 31	3 29	11 11	6 33	29 36	0 091	0 032
2 56 " - 5 56 "	0 763	0 254	5 32	1 77	6 97	2 60	12 06	0 014	0 005
5 56 " - 9 19 "	0 756	0 224	3 91	1 16	5 18	1 22	5 66	0 113	0 034
9 19 " -11 57 "	0 695	0 264	4 17	1 58	6 00	1 69	7 84	0 165	0 049
11 57 " - 5 58 a.m.	1 328	0 221	7 96	1 33	5 99	3 23	14 98	0 439	0 073
May 28 5 58 a.m.- 9 07 a.m.	0 720	0 229	3 80	1 21	5 27	1 24	5 75	0 340	0 108
9 07 " - 3 06 p m.	1 770	0 296	7 16	1 20	4 04	0 86	3 99	1 178	0 197
						21 56	= 100 00	4 046	0 237
3 06 p.m.- 8 09 a.m.	5 454	0 320	19 36	1 14	3 55				
May 29									

Recovery of extra sugar 21.56 gm = 74.3 per cent on the basis of D·N = 3.56

TABLE VIII.
Experiment 9.

Dog 4. 8th to 10th phlorhizin day Weight 10.0 kilos.

Date and time	Urine									
	Nitrogen		Sugar		D N ratio	Extra sugar		Acetone bodies		
	Total	Per hr	Total	Per hr		Total	Per hr	Total	Per hr	
	gm	gm	gm	gm		gm	gm	gm	gm	
1925 Apr 13 6 20 p m - 9 20 p m. 9.23 p m.	1 523	0 508	4 89	1 63	3 21			0 281	0 094	
30 gm of glucose by stomach tube										
Apr 13-14 9 20 p.m - 12 21 a m 12 21 a m - 3 23 " 3 23 " - 6 20 " 6 20 " - 9 20 " 9 20 " - 12 20 p.m	1 362 1 319 1 026 1 008 1 033	0 451 0 435 0 348 0 336 0 344	11 40 13 17 6 48 5 62 3 92	3 78 4 34 2 20 1 87 1 31	8 37 9 99 6 31 5 58 3 79		7 04 8 95 3 20 2 40 0 71	31 57 40 14 14 35 10 76 3 18	0 128 0 027 0 0 015 0 041	0 042 0 009 0 0 005 0 014
12 20 p.m - 3 21 p m 3 21 " - 6 20 " 6 20 " - 11 20 " 11 20 " - 3 51 a m Apr 15 3 51 a m - 6 52 a m.	1 474 1 156 2 174 1 996*	0 489 0 388 0 435 0 442	4 33 2 93 4 14 3 45	1 43 0 98 0 83 0 77	2 93 2 53 1 90 1 73		22 30	100 00	0 088 0 080 0 100 0 230*	0 029 0 027 0 033 0 051
	1 347	0 447	4 28	1 42	3 18				0 120	0 040

Recovery of extra sugar 22 30 gm. = 74.3 per cent on the basis of D N = 3.21

* Interpolated on the basis of D N which was determined in the sample collected from the second half of this period.

TABLE IX
Experiment 10
 Dog 12. (Second phlorhizinization) 8th to 10th phlorhizin day Feb. 22, weight 13.5 kilos

Date and time	Urine										Blood	
	Nitrogen		Sugar		D N ratio	Extra sugar			Acetone bodies		Sugar	CO ₂ - combin- ing power
	Total	Per hr	Total	Per hr		Total	Per hr	Total elimi- nated	Total	Per hr		
	gm	gm	gm	gm		gm	gm	per cent	gm	gm	per cent	sols per cent
1926 Feb. 22 12 12 p.m.- 3 17 p.m.	1 176	0 382	4 39	1 43	3 74				0 791	0 257	0 047 3 00 p.m.	38
3 19 p.m.	31.41 gm of glucose by stomach tube (= 2.33 gm per kilo of body weight)											
3 17 p.m.- 6 17 p.m.	0 913	0 304	10 25	3 42	11 23	6 83	2 28	23 01	0 365	0 122	0 063 4 25 p.m. 0 152	38 43
6 17 p.m.- 9 17 p.m.	0 727	0 242	14 29	4 76	19 64	11 57	3 86	38 97	0 112	0 037	6 05 p.m. 0 145	52
9.17 " -12.17 a.m.	0 625	0 208	6 01	2 00	9 62	3 67	1 22	12 37	0 049	0 016	8 15 p.m. 0 074 11 10 p.m.	52
Feb. 23 12 17 a.m.- 3 17 a.m.	0 691	0 230	3 51	1 17	5 08	0 93	0 31	3 13	0 074	0 024	0 042 1 20 a.m.	48

3.17 a.m.- 6 17 a.m.	0 694	0 231	4 75	1 58	6 84	2 15	0 72	7 24	0 113	0.038	0 056 4 20 a.m.	45
6.17 " -10.05 "	0 888	0 234	6 01	1 58	6 76	2 69	0.70	9 06	0 206	0 054	0.054 8 00 a.m.	37
10.05 " - 4 39 p.m.	1 752	0 267	8 39	1 28	4 79	1 84	0 28	6 20	0 801	0 122		
Feb. 23-24	6 290		53 21			29 68	=	100 00				
4 39 p.m.- 8 45 a.m.	5 237	0 325	18 85	1 17	3 60				3 617	0 258		

Extra sugar recovery (D:N 3 74) given 31 44
 recovered 29 68
94 41 per cent.

Extra Sugar Recovery.

Extra sugar in the urine was calculated on the basis of the initial D:N ratio². Out of 232.09 gm. of glucose given altogether 189.11gm. were recovered in the urine,³ that is to say, 81.5 per cent. As may be seen from Table X, Columns 3 and 4, the D:N ratio returned at the end, in most of the experiments, to almost exactly the same level as it was at the beginning. Experiment 9 is exceptional in this respect, as the D:N ratio dropped towards the end of the experiment as low as 1.73 (Table VIII), hand in hand with an extremely low total sugar (about 50 per cent lower than in the initial period).

Recovery of extra sugar seems not to depend to any great extent on the height of the initial D:N ratio. In Experiment 7, recovery was 74.3 per cent on the basis of $D:N = 3.56$, and in the same dog (Experiment 9) on the basis of $D:N = 3.21$, recovery was exactly

² Calculated according to the formula

Extra sugar = (total sugar of the given period) - (nitrogen of the given period \times initial D N ratio)

That is also the definition of what we mean here by extra sugar (Lusk). This calculation of extra sugar in feeding experiments with phlorhizinized dogs is based on the assumption that after feeding the diabetic disturbance of the metabolism of the given animal remains the same as before feeding and that therefore the endogenous D:N ratio (in contrast to the exogenous D:N ratio when sugar-forming and nitrogen-containing substances are given to such an animal) in the urine may be assumed as constant during such an experiment. Present work however shows that changes develop in the metabolism of a phlorhizinized dog after the feeding of glucose, therefore all such calculations intended to find out the extent of sugar formation from a certain sugar-forming substance must be taken with caution.

³ Two experiments are not recorded here. The *first* one with 20 gm. of glucose was not conducted in the usual way. It was made on the 15th day of phlorhizinization, the basal D:N ratio was 2.7 and only one sample of 24 hours duration was obtained after the feeding of glucose, no sample from the after period being obtained. During the time this sample was being collected the dog had convulsions, was in a condition of collapse, and the extra sugar recovery was 106 per cent. In the *second* experiment, in which 10 gm. of glucose were given, the dog had fever, ketosis was extremely small, and the D:N ratio showed a drop at the end of the experiment of such magnitude that the calculation of extra sugar became a matter of playing with the D:N ratios (in such a way the sugar recovery was either 53 or 84 per cent).

TABLE X
Summary of the Quantitative Relationships Existing in the Metabolism of Phlorizinized Dogs after Ingestion of Glucose. Table Arranged According to the Increasing Amounts of Glucose Given.

(1) Experiment No	(2) Glucose given gm	D N ratio		Glucose recovered		(7) Glucose unrecovered gm	(8) Nitrogen spared during whole experiment gm	Ketolytic action			
		(3) Initial	(4) Final	(5) Amount gm	(6) Percent of amount given			(9) Original amount of ketone bodies which disappear calculated as acetone	(10) Corrected by re- duction to com- mon preliminary level	(11) Correction factor	(12) Ketolytic action of 1 gm of ingested glucose ((10) ÷ (12))
1	10 00	3 19		7 73	77 3	2 27	0 40	0 54	1 13	2 1	0 113
3	15 00	3 17	3 15	13 56	90 4	1 44	0 88	1 70	1 55	0 91	0 103
4	19 34	3 40	3 14	14 63	75 7	4 71	0 72	2 38	2 86	1 2	0 148
5	19 34	3 92	3 79	15 57	80 5	3 77	1 46	1 70	1 62	0 95	0 084
2	20 00	3 19		18 95	94 8	1 05	1 39	0 99	1 51	1 5	0 076
6	29 00	2 97	3 06	20 71	71 4	8 29	2 08	1 47	3 01	2 1	0 104
7	29 00	3 56	3 55	21 56	74 3	7 44	2 43	4 60	3 54	0 77	0 122
8	29 00	3 14	3 15	24 42	84 0	4 58	2 75	2 56	3 33	1 3	0 115
9	30 00	3 21	2 93	22 30	74 3	7 70	*	*			
10	31 41	3 74	3 60	29 68	94 4	1 73	2 68	4 79	3 74	0 78	0 119
	232 09	3 35	3 30	189 11	81 5	42 98					0 109
	Sum.	Average	Average	Sum	Average	Sum					Average

* Could not be determined because of large discrepancy between the initial and final levels.

the same; *i.e.*, 74.3 per cent with almost identical amounts of glucose. In Dog 12 on the basis of $D:N = 3.17$, recovery was as high (90.4 per cent, Experiment 3) as on the basis of 3.74 (94.4 per cent, Experiment 10) with different amounts of glucose. During the second phlorhization in one experiment as much extra sugar was recovered as during the first with the same amounts of glucose (Experiments 7 and 9). Smaller amounts of glucose may be recovered as well as larger; with 10 to 15 gm. 77 to 90 per cent, with 30 gm. 71.4 to 94.8 per cent.

Total Sugar Excretion as Compared with Extra Sugar Elimination.

Hourly total sugar elimination (fifth column, Tables I-IX, XI) comes back to the preliminary level before all extra sugar (seventh column, Tables I-IX) has been excreted, as may be seen from Table XIII. The significance of this fact will be clear from the following paper.

Respiratory Quotient in Completely Phlorhizinized Dogs after Glucose Feeding.

Much effort has been expended in this work to solve the question whether or not glucose may be oxidized in completely phlorhizinized dogs. It has been at the cost of vague interpretations (3) and by abstinence from premature conclusions (4) that the complete explanation was obtained after the last step had been made and the respiratory metabolism of phlorhizinized dogs after glucose feeding examined. It appeared as a logical consequence and final proof of the whole work that unmistakable rises in *r.q.* were found (1) after the feeding of 20 gm. of glucose or more in phlorhizinized dogs (showing in fasting all the signs of complete phlorhization: proper *D:N* ratio, diabetic *r.q.* of 0.70, and high ketosis), provided the dogs were put in the calorimeter soon enough after glucose ingestion. These rises in *r.q.* were high enough to account for most if not all of the unrecovered sugar. This calorimeter work showed that the chief criteria of the completeness of phlorhizin diabetes at present available, *i.e.* *D:N* ratio around 3.6, *r.q.* of 0.70, and high ketosis, can be considered only as correct criteria of the completeness of phlorhization, but cannot be considered as evidence of the complete inability of the animal to oxidize glucose.

Nitrogen-Sparing Action.

Nitrogen excretion in the dogs of this series was in fasting animals 0.028 to 0.064 gm. of N per kilo of body weight per hour. After ingestion of glucose, in all the experiments a decrease in nitrogen excretion was found, having all the signs of suppression of the metabolism of nitrogen-containing substances (proteins), and not retention of nitrogen in the body. Table XI has been constructed to show (besides other facts) the course of nitrogen excretion expressed in percentage of the preliminary level. The lowest point with 10 gm. is 88 per cent, with 15 gm. 75 per cent, with 20 gm. 66 to 82 per cent, and with 30 gm. 54 to 71 per cent of the basal amount. In order to get a complete quantitative expression of the nitrogen-sparing action, in every single experiment corresponding areas included between the curve of nitrogen excretion and the straight line joining the preliminary and final levels of nitrogen excretion were planimetered, and in such a way one value was obtained representing with accuracy the nitrogen spared in one figure. It can be seen from Table X, Column 8, and from Chart 1, that the amount of nitrogen spared increases nearly proportionately with the increasing amount of glucose given (Table X, Column 2, and Chart 1), but is not parallel to the amount of unrecovered glucose (Table X, Column 7).

Ketolytic Action.

It is striking how the curve of ketone body excretion follows the curve of nitrogen excretion (Table XI). This fact has been noticed before by several authors who have determined these urine constituents over longer periods. Here, however, the same parallelism occurs during examination of the urine in 3 hour periods. The lowest points of both curves appear almost at the same time from 3 to 12 hours after ingestion of different amounts of glucose. The larger the amount of ingested glucose is, the later the lowest point appears. At this time the urine either does not give any acetone reaction at all or shows only minimal traces of it (with larger amounts of glucose). The ferric chloride test is negative always before Legal's test and positive after it. Clinical symptoms will be discussed in the following paper as well as the behavior of the CO_2 -combining power of the blood, and ketone bodies in the blood and breath.

TABLE XI.
Comparison of Total Sugar, Nitrogen, and Ketone Body Excretion in Phlorhizinized Dogs after Glucose Ingestion.

Experiment No	Glucose fed	Eliminated in urine	Excretion per hr expressed in terms of percentage of initial excretion of substance, assumed as 100 per cent															
			Preliminary.	3 hrs	6 hrs	9 hrs	12 hrs.	15 hrs	18 hrs	21 hrs	24 hrs	27 hrs	30 hrs	33 hrs	36 hrs	39 hrs	42 hrs	
1	10 00 gm.	Total sugar	100*	180	102	90	100											
		Nitrogen	100*	96	91	88	100											
		Ketone bodies	100	51	26	89	137											
3	15 00	Total sugar	100	269	160	98†	99	104										
		Nitrogen	100	87	75	83†	93	104										
		Ketone bodies	100	38	10	16†	56	87										
4	19 34	Total sugar	100	254	271	120	89	87	93	82	85	85	85					
		Nitrogen	100	97	79	66	72	78	96	87	93	93	93	93				
		Ketone bodies	100	61	9	3	11	35	59	52	79	79	79	79				
5	19 34	Total sugar.	100	318	185	122	104	79	72	92	87							
		Nitrogen	100	88	79	74	90	92	77	86	88							
		Ketone bodies	100	33	13	24	71	101	103	116	123							
2	20 00	Total sugar.	100	147	167	137	97	105	105	112	110							
		Nitrogen	100	95	82	86	85	94	94	99	99							
		Ketone bodies.	100	91	5	4	74	115	104	111	76							

6	29 00	Total sugar. Nitrogen. Ketone bodies	100 100 100	355 88 41	406 61 10	114 54 13	99 58 22	85 61 30	80 61 46	82 64 71	99 75 113	104 85 128		90 88 135		101 101 101	101 113 92	
7	29 00	Total sugar. Nitrogen Ketone bodies.	100 100 100	240 118 71	291 94 12	157 80 2	103 71 13	140 84 19	118† 70† 28†	107 72 42	106 94 76	106 94 76	101 113 92	101 113 92		101 101 101	101 113 92	
8	29 00	Total sugar. Nitrogen Ketone bodies	100 100 100	380 92 45	380† 92† 45†	280 68 3	101 57 3	99 62 3	92 73 5	65 72 10	60 69 29	75 79 54	83 79 77	*83 79 77	93 87 58		93 87 58	
9	30 00	Total sugar. Nitrogen Ketone bodies	100 100 100	232 89 45	266 86 10	135 69 0	91 66 5	80 63 15	88 96 47	60 76 29	51 86 35	51† 86† 35†	47† 87† 54†	87 88 43				
10	31 41	Total sugar. Nitrogen Ketone bodies	100 100 100	239 80 47	333 63 14	140 54 6	82 60 9	111 60 15	111† 61† 21†	90 70 47	90 70 47	82 85 100	82 85 100	82 85 100	82 85 100		82 85 100	

* Instead of the preliminary figures, the final levels of glucose and nitrogen excretion were used in this particular experiment

† Period a little longer or shorter than 3 hrs.

The area included by the curve of ketone body excretion has been determined in the same way as with nitrogen, and the original figures are contained in Table X, Column 9. If these original figures of ketolytic action are compared with the figures of nitrogen-sparing action (Table X, Column 8), only rough parallelism between them can be observed. It has been noticed, however, that the higher the initial level of ketone body excretion, the greater is the effect of the same amount of glucose. For instance, in Experiments 6 and 7 the weight of the dogs was almost the same, but in Experiment 6 (Table V) the dog excreted 0.097 gm. of ketone bodies (calculated as acetone) per hour, while in Experiment 7 (Table VI) 0.258 gm. was excreted. After administration of identical amounts of glucose (29 gm.) the amount of ketone bodies which disappeared (Table X, Column 9) was in the first case 1.47 gm. and in the latter 4.60 gm., the proportion being 1:3, as in the preliminary ketone body excretion. On the other hand, in the dogs having a similar level of initial ketone body excretion, the same amount of glucose causes a similar effect. For instance, in Experiments 4 and 5 (Tables III and IV) in different dogs ketone bodies were excreted in the preliminary sample of urine at the rate of about 0.20 gm. per hour, and the ketolytic effect (Table X, Column 9) was around 2.0 gm. in both experiments (2.38, 1.70). In Experiments 7 and 10 ketone bodies were excreted at the rate of 0.26 gm. per hour (Tables VI and IX) before glucose ingestion and equal amounts of glucose caused the disappearance of 4.60 and 4.79 gm. of ketone bodies (Table X, Column 9). After that was noticed, all the figures obtained in Table X, Column 9, were recalculated on the following basis. A standard preliminary ketone body excretion level was chosen as being 0.20 gm. of ketone bodies per hour (it is immaterial what figure we take as standard). Afterwards 0.20 was divided by the figure representing initial ketone body excretion per hour as actually obtained in each experiment, and thus a factor was secured which is recorded in Table X, Column 11, by which the figures contained in Column 9 were multiplied, giving the values in Column 10. It can distinctly be seen that the ketolytic effect (Table X, Column 10) after this correction is in most cases fairly parallel to the amount of glucose given (Column 2) and to the nitrogen spared (Column 8). This can clearly be seen from Chart 1. It shows no paral-

lelism, however, with the amount of unrecovered glucose (Column 7) or with the percentage of recovery (Column 6). After this correction ketolytic action of 1 gm. of ingested glucose (Table X, Column 12, also Chart 1) amounts to about 0.1 gm. of acetone as an average in different experiments.

One more regularity (which has not been noted so far) occurs in almost all the experiments (Table XI); namely, that the nitrogen and ketone body excretion drops as long as total sugar excretion stays above the preliminary level. As soon as the latter

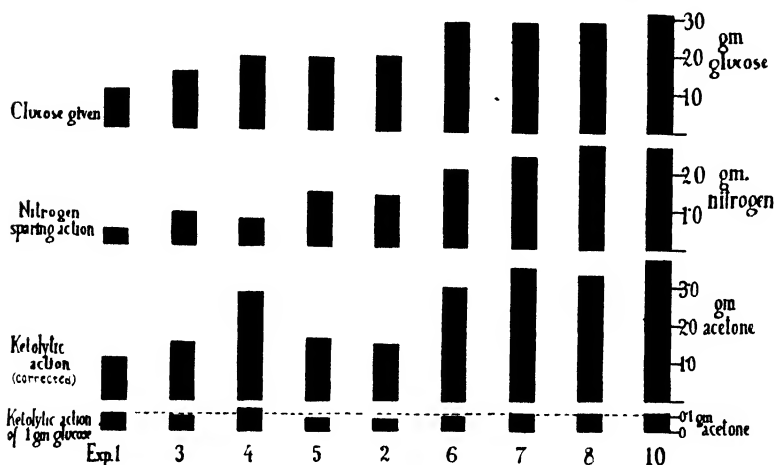


CHART 1. Quantitative relationships existing in the metabolism of phlorhizinized dogs after ingestion of glucose. Dotted line represents average of the ketolytic action of 1 gm. of glucose.

reaches the basal level or drops below it, both these urine constituents begin to increase, and the favorable influence of glucose is over (see Paper III).

The lowest point of ketone body excretion (Table XI) amounted with 10 gm. of glucose to 26 per cent, with 15 gm. to 10 per cent, with 20 gm. to 3 to 13 per cent, and with 30 gm. to 0 to 6 per cent of the amount excreted in the first instance.

Adrenalin and Ketosis.

Dog 4 was subjected to two phlorhizinizations. During the first phlorhizinization as an exception adrenalin was used, two

subcutaneous injections of 0.4 mg. each on the 1st day, one more on the next day. 30 hours after the first phlorhizin injection the D:N ratio was already 3.14 (during the 1st day it was 6.68) and with this D:N ratio a typical semicomatose picture appeared with strong acetone odor from the mouth, vomiting, and slight convulsions, and the next day, some 48 hours after the start of phlorhizination, Kussmaul breathing with a D:N ratio of 3.38. In order to save the dog, glucose was given (Table I, Experiments 1 and 2). In the *second* phlorhizination, which was conducted without the use of adrenalin, the D:N ratio on the 5th day was still 4.06 and the dog was in relatively good condition. The criticism might be applied to this experiment, that on those two occasions the dog was getting different food before the start of the experiment: poor in the first instance and rich in the second. No one of the dogs, however, so far examined in this series without adrenalin showed the signs of coma so quickly as this one with the additional use of the drug. Adrenalin was not used any more.

*Influence of the Ingestion of Fat on Metabolism of a
Phlorhizinized Dog.*

It has been showed by Fejes (5) that after ingestion of butter fat by a phlorhizinized dog the production of ketone bodies increases. The experiment recorded in Table XII was made⁴ in order to show whether fat is really a ketogenic factor in the phlorhizinized dog. After ingestion of 100 gm. of olive oil by a phlorhizinized dog some of it was certainly absorbed because strong lipemia ensued; the rest was eliminated in the feces, but nothing was vomited. Ketone body excretion was doubled, with CO₂-combining power showing no important change. The interesting feature of this experiment is the tendency of the D:N ratio to fall progressively due to the decrease of total sugar excretion going hand in hand with blood sugar depression. At the lowest blood sugar level of 0.041 per cent, we would have expected convulsions. They did not appear, however. Nitrogen was not spared. The body began to "smoke with acidosis compounds" (Woodyatt (6)).

⁴ At the suggestion of Prof S R Benedict.

TABLE XII
Ketogenic Influence of Ingested Olive Oil in Phlorhizinized Dog.

Dog 14 8th phlorhizin day Feb 12, weight 7.6 kilos Water in constant quantity supplied every 3 hrs. by stomach tube.			Urine						Blood		
			Nitrogen		Sugar		D N ratio	Ketone bodies as acetone		Sugar	CO ₂ -combining power
								Total	Per hr		
			Time	Total	Per hr	Total	Per hr		Total	Per hr	
1926			gm	gm	gm.	gm	gm		gm		
Feb 12	2 33 p m - 5 33 p m	1 013	0 344	4 23	1 41	4 18	0 268	0 089	0 059 5 20 p m	46	
5.35 p.m.	100 gm. of olive oil by stomach tube										
5 33 p m - 8 41 p m	0 955	0 305	4 13	1 38	4 32	0 249	0 079	0 055 7 33 p m	47		
8 41 " - 11 34 "	0 955	0 331	3 57	1 24	3 74	0 311	0.108 0 050	9 33 p m 0 048	48 Lipemia.		
11 34 " - 2 39 a m	1 139	0 369	3 96	1 28	3 48	0 524	0.170 11 40 p m		49 "		
Feb 13											
2 39 a m.- 5 35 a m	0 970	0 331	3 17	1 08	3 27	0 514	0.175 0 041	0 048 1 40 a m 0 041	49 Lipemia		
5 35 " - 8 35 "	1 118	0 373	3 43	1 14	3 07	0 550	0 183	3 40 a.m.	48		
Average	0 321										

DISCUSSION.

It would be futile to try to review here all the extra sugar recovery experiments published so far (7-15). These experiments although all equally reliable, are not comparable with one another, since they were made by different workers and methods, with different purposes in mind, some of the results being selected and published as more successful than the others considered unsuccessful and therefore discarded. All the correct experiments made in this series are recorded here. Under the conditions described I could not recover in the urine all the glucose given and that is a fact of importance. No direct attempt was made by me

TABLE XIII

Experiment No	Glucose intake	Total sugar excretion above basal level Hrs after feeding	Extra sugar elimination Hrs after feeding
	<i>gm</i>		
1	10 00	3	12
3	15 00	6	16
4	19 34	9	15
5	19 34	9	12
2	20 00	9	24
6	29 00	9	27
7	29 00	9	30
8	29 00	9	15
9	30 00	9	15
10	31 41	9	25

to show that this unrecovered glucose was lost irreversibly, but it must have been in accordance with the whole picture.

If we try to extrapolate this extra sugar recovery by using the figures of other authors obtained with amounts of glucose smaller and larger than these which have been applied here, with the same criticism in mind however as mentioned above, we will find that Ringer and Frankel (9) with 9 gm. of glucose obtained 75.2 and 96.8 per cent, therefore on an average 86 per cent of extra sugar recovery, Nash (13) with 20 to 60 gm. 91 per cent,⁵ Ringer (15) in one experiment with 75 gm. 92 per cent⁶ and in another one with

⁵ Nash's experiments were made in earlier days of phlorhization than the present experiments

⁶ Calculated on the basis of full basal D N ratio without omission of the second decimal

150 gm. 86 per cent.⁶ The metabolic effect of the ingested glucose may be proved in all these experiments as well with small amounts of glucose as with larger ones, and in the present series is proportional to the total amount of glucose ingested and not to the amount of unrecovered glucose. This fact is surprising but it may possibly be explained in the following simple way. With different amounts of glucose a certain fraction of the ingested sugar is always utilized in a phlorhizinized dog. Simultaneous extra sugar recovery in the urine, however, being a result of a calculation, based on the D:N ratio (which is composed of two variables) is subject to a number of endogenous influences and may therefore to a certain limited extent go in one or the other direction *in part independently from the extent of the utilization of glucose*. Only in such a way also may the paradoxical fact be brought into agreement with the present work, that sometimes more sugar may be recovered in the urine than was ingested, although there were convincing proofs indicating that some of the glucose ingested must have been oxidized as was observed by Deuel and Chambers (12) and others. In such a way the fundamental relationship would become evident which apparently exists in a completely phlorhizinized dog between the nitrogen-sparing and ketolytic action on one hand and glucose utilization on the other.

Objection has been raised by several authors (Ringer, Nash) that from the dynamogenic standpoint the large metabolic effect of the unrecovered glucose cannot be explained adequately by the oxidation of this small amount of glucose. It is however relatively easy to answer. Small amounts of oxidizing glucose induce complete oxidation of ketone bodies and these two foodstuffs spare protein. We must consider the fact that protein in phlorhizin diabetes gives off very little energy in metabolic processes, more than half of it being eliminated as sugar and of the rest a certain part possibly converted into ketone bodies. In such a way large amounts of protein may be spared by some other foodstuff, if the last one only oxidizes in a proper way.

Table XIV may help us to consider the matter quantitatively. Let us assume that the calorie production of the animal remains constant after ingestion of glucose. Caloric value of protein spared in nine experiments is 407 calories. From this amount should be subtracted the number of calories which would possibly

be wasted as glucose, in case this protein were metabolized (58 per cent out of 92.5 gm. of protein spared should be multiplied by the caloric value of glucose (1 gm. = 3.755 calories) and subtracted from 407 calories). The remaining 171 calories should not necessarily all be replaced by some other foodstuff because one part might have been wasted as ketone bodies. Now let us assume for this particular purpose that unrecovered glucose represents really glucose utilized, and then that would represent 132.5 calories. Complete oxidation of the β -hydroxybutyric acid as result of ketolytic action of glucose amounts to 174.4 calories.⁷ As we see, there is no trouble in finding out what has replaced in the metabo-

TABLE XIV

Dynamogenic Balance after Glucose Ingestion in Phlorhizinized Dogs.

Table No	Sugar administered	Protein spared		Sugar unrecovered supposedly oxidized		β -hydroxybutyric acid supposedly oxidized	
		gm	cals	gm	cals.	gm	cals
I	10 00	2 5	11 0	2 27	8 52	0 95	4 46
III	15 00	5 5	24 2	1 44	5 41	3 05	14 31
IV	19 34	4 5	19 8	4 71	17 68	4 27	20 02
V	19 34	9 1	40 0	3 77	14 16	3 05	14 30
II	20 00	8 7	38 3	1 05	3 94	1 78	8 33
VI	29 00	13 0	57 2	8 29	31 13	2 64	12 36
VII	29 00	15 2	66 9	7 44	27 94	8 25	38 69
VIII	29 00	17 2	75 7	4 58	17 20	4 59	21 53
X	31 41	16 8	13 9	1 73	6 50	8 59	40 28
Total		92 5	407 0	35 28	132 48	37 17	174 38

lism the spared protein. In fact it looks as if there were apparently an excess of energy in our calculation (306.9 calories from glucose and β -hydroxybutyric acid for 171 calories from protein). But this is not the case because our figure showing the ketolytic effect does not represent ketone bodies which were necessarily oxidized. Ketolytic effect may be due here to several reasons: (1) Protein metabolism was depressed and less ketone bodies liberated from it. (2) Oxidizing glucose replaced the fat in metabolism and thus less fat was metabolized and fewer ketone bodies produced.

⁷ Obtained by the recalculation of the figures for ketolytic action in Table X, Column 9, expressed as acetone into β -hydroxybutyric acid and by multiplying this by the caloric value of 1 gm. of β -hydroxybutyric acid.

These two reasons may have been sufficient to account for one part of the ketolytic effect. The last reason, however, seems to play the chief rôle, and that is (3) oxidizing glucose helped to catabolize ketone bodies by direct chemical influence in the sense of Shaffer (16). In such a way a balance of energy exchange in such experiments may be obtained in a rough way. Acetone eliminated in the breath of these dogs was not taken into account here.

The present work explains the mechanism of action of glucose on phlorhizin ketosis on the basis of the well known and acknowledged views.

SUMMARY.

1. A number of completely phlorhizinized dogs was given from 10.00 to 31.41 gm. of glucose. In ten experiments, 81.5 per cent of the glucose given was recovered as extra sugar in the urine, showing that less sugar is excreted than is fed.

2. The total sugar elimination after feeding glucose reaches the initial level before all extra sugar has been excreted, and it is total sugar elimination not extra sugar excretion with which nitrogen and ketone body excretion seems to be in relationship.

3. After glucose ingestion, constant nitrogen-sparing action was observed in the shape of a regular curve. The quantities spared amounted to 0.40 to 2.75 gm. of nitrogen, corresponding to 2.5 to 17.2 gm. of protein with different amounts of glucose. The nitrogen-sparing action is proportional to the amount of glucose given and not to the unrecovered glucose.

4. Parallel with nitrogen-sparing action, ketone body excretion decreased and increased again, as observed in 3 hour samples of urine. The amount of ketone bodies which disappeared corresponded to 0.95 to 8.57 gm. of β -hydroxybutyric acid.

5. The preliminary level of ketone body excretion seems to play an important rôle in the extent of the total ketolytic effect. If a simple correction is used, reducing all the preliminary levels of ketone body excretion in different dogs to one common level, and this correction is applied to the total ketolytic effect in every experiment, a fair parallelism may be obtained between the amount of glucose given and the ketolytic effect, but not between the latter and the amount of the unrecovered glucose. In this way the keto-

lytic action of 1 gm of ingested glucose amounts to 0.1 gm. of acetone as the average in different experiments.

6. In one experiment in which adrenalin injections have been added to the usual procedure, coma was produced 36 hours after the first phlorhizin injection.

7. Ingestion of olive oil by a phlorhizinized dog gives rise to the increased production of ketone bodies

8. All the phenomena observed in connection with the rise in respiratory quotient, obtained in calorimeter work (1), and with the blood sugar studies, contained in the following paper, represent all our criteria (available at the present time) for the oxidation of glucose, and prove that in completely phlorhizinized dogs after ingestion of glucose, small amounts of it are oxidized, a fact which has never been proved conclusively so far.

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INTERMEDIARY CARBOHYDRATE METABOLISM.

III. VITAL ACTION OF GLUCOSE IN PHLORHIZIN DIABETES.

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The purpose of the preceding paper was to show the quantitative relationships which exist in the metabolism of a phlorhizinized dog after ingestion of glucose. In this paper the vital rôle will be pictured which isolated carbohydrate metabolism plays in the organism of these dogs by keeping away two intricately associated syndromes: (1) hypoglycemia with convulsions and (2) ketosis with coma, either of which or both may cause death.

Method.

Clinical observations and blood studies were made on the series of dogs used in the preceding paper, at the point they went into convulsions or coma. Blood was drawn in these dogs from one of the saphenous veins which was exposed under novocaine anesthesia before the experiment. Methods used were the Shaffer-Hartmann for blood sugar, Van Slyke's constant pressure method for CO₂-combining power of the blood, and Van Slyke and Fitz' for total ketone bodies of the blood. All determinations except those of the ketone bodies were made in duplicate. As the animals were weak after long fasting and phlorhization larger samples of blood required for duplicate determination of ketone bodies could not be drawn.

Results.

Convulsions.

These appeared spontaneously in six out of sixteen phlorhizinized dogs observed. They appeared first in a typical manner as follows: small trembling movements of the head, which sometimes

stopped by themselves, or increased with the course of hours, the dog being still completely conscious, but showing extreme weak-

TABLE I
Convulsions in Phlorhizinized Dogs.

Dog No	Day of phlorhization	Blood sugar	D/N ratio in 3 hr samples of urine	Ketone bodies in the urine per hr	R Q	Onset of convulsions
		<i>per cent</i>		<i>gm</i>		
5	5	0 048	3 29 3 23 3 08	0 117 0 106 0 159		15 hrs after ingestion of 20 gm. glucose.
4	4		3 65 3 69	0 136 0 146		18 hrs. after ingestion of 20 gm glucose.
4	15			+++		16 hrs. after ingestion of 20 gm glucose.
12	8	0 047	3 74	0 257		In fasting
8	7		3 18	0 076	0 700 (average for 3 hrs)	In fasting.
13	4		3 79	+++	0 699 (average for 2 hrs)	In fasting.
4	14	0 060 0 056	3 03	+++ +		In fasting Returned 9 hrs. after ingestion of 30 gm. glucose
9	9	0 062 0 051		+++ +++		In fasting Returned 11 hrs after ingestion of 30 gm. glucose
			3 41 average			

ness and apathy. In this condition, the symptoms resembled those of insulinized dogs. Sometimes opisthotonos appeared.

A synopsis of the condition of these dogs when convulsions appeared first is given in Table I.

As may be seen from Table I, these dogs presented apparently the picture of the most severe type of diabetes, showing in fasting a D:N ratio from 3.03 to 3.79, respiratory quotient of 0.70,¹ strong (in fasting) or relapsing (after ingestion of glucose) ketosis. Blood sugar was between 0.047 and 0.062 per cent.

Convulsions occurred in fasting and phlorhizinized dogs. In Dog 4 they occurred in both phlorhizinizations. In three instances, however, they appeared for the first time 15 to 18 hours after ingestion of glucose. In Dog 13 they appeared during the 4th day of phlorhization, were allowed to last until the 5th day, and then the dog died giving a picture of some kind of delirium with running movements of the legs while lying on the floor, with unconsciousness, and strong ketosis. Glucose administered to this dog half an hour before death was of no avail. The symptoms had evidently progressed too far. In other cases, however, glucose or meat administered promptly relieved the convulsions, as shown in the following two experiments (Tables II and III).

In the experiment in Table II the convulsions stopped in 21 minutes, in that in Table III in 56 minutes after ingestion of glucose. After meat ingestion, however, the convulsions stopped almost immediately (a few minutes after ingestion). If the convulsions were stopped by glucose they would reappear in exactly the same form as they appeared first before the glucose feeding, as soon as the glucose action was over, as indicated by the blood sugar curve which at this point would drop below the level observed in the first instance. (See Tables II and III.) The curve of CO₂-combining power of the blood did not follow exactly the blood sugar curve in these two experiments, but rose more quickly and fell more slowly.

Convulsions which appeared in this series of dogs were evidently hypoglycemic convulsions. They were first described in phlorhizinized Eck fistula dogs by Erdélyi (1) and Burghold (2) and recently by Fischler and Ottensooser (3) and the author (4) in fasting phlorhizinized animals. They belong thus to the same group of convulsions as appear in hepatectomized dogs (Mann (5))

¹ Determined in Cornell University calorimeter in the Department of Physiology of Cornell University Medical College.

and insulinized animals (6). Phlorhizin used in this work was Merck's product, the purest on the market, recrystallized. According to Professor S. R. Benedict,² however, in his dogs phlorhizinized with Kahlbaum's phlorhizin, which contains asymmetric phlorhizins, most violent convulsions may be observed which are

TABLE II

Dog 4 14th phlorhizin day. Weight 7.4 kilos

Time	Blood sugar	Breath acetone	Urine acetone	Condition of dog
<i>1925</i>	<i>per cent</i>			
June 3				<i>Intermittent convulsions of the neck muscles. Opisthotonos.</i>
11 00 a m				Urine $\frac{D}{N}$ 3 03 (11 08 a m.-12 42 p m.).
12 42 p m	0 060	+	++	<i>Convulsions.</i>
12 44 "	30 gm of glucose by stomach tube			
1 05 "				Abrupt end of convulsions
1 44 "	0 126	+		No convulsions
2 44 "	0 174	+		" "
3 44 "	0 190	+		" "
4 44 "	0 176	?		" "
5 44 "	0 164	0	+	" "
6 44 "	0 131	0		" "
7 44 "	0 082	0	Slight trace	" "
8 44 "	0 068	0		" "
9 44 "	0 053	0		" "
10 03 "		0	Trace	<i>Convulsions of the neck muscles. Opisthotonos.</i>
10 44 "	0 056			Prompt recovery.
	200 gm. of meat.			

not stopped by glucose ingestion, and which finally cause the death of the animal.

Moreover, while convulsions were being stopped by glucose, the other component of the clinical picture, namely ketosis, was undergoing changes as well.

² Personal communication.

TABLE III.

Vital Action of Glucose on Convulsions and Acidosis of a Phlorhizinized Dog.

Dog 9. 9th phlorhizin day. Weight 13.5 kilos.

Time.	Blood sugar	CO ₂ -combining power	Breath acetone	Condition of dog
<i>1925</i>	<i>per cent</i>	<i>vols per cent</i>		
July 1 10 00 a m.			++	Dog barking, howling. Torsions of the whole body, <i>opisthotonos</i> , strong reflex irritability (as during strychnine poisoning). Afterwards <i>convulsions of the neck muscles</i> . Finally dog unconscious, insensitive to skin prick.
11 07 "	0.062	50	++	
11 12 "	30 gm of glucose by stomach tube.			
11 17 "				Convulsions stop and reappear every few minutes until 12 08 p m.
12 08 p m				<i>Convulsions stop definitely.</i>
12 20 "	0.130	60	++	Reflex irritability gone. Sensitivity to pain reappears.
12 45 "				Gets up to pant. Angry, biting when position is uncomfortable.
1 12 "	0.149	59	++	Panting
1 40 "				Stronger. Jumped down from the board, walked around the room and urinated
2 12 "	0.167		+ Trace	
3 12 "	0.153	60		<i>Walking, active.</i>
4 12 "	0.101	60	0	
5 12 "	0.084		0	Sits quietly.
6 12 "	0.084	63	0	Catching flies.
7 12 "	0.076	56	0	Quiet
8 12 "	0.073	58	0	Sits quietly.
9 12 "	0.066	53	Trace.	Weaker

TABLE III —*Concluded*

Time	Blood sugar	CO ₂ -combining power	Breath acetone	Condition of dog
<i>1920</i>	<i>per cent</i>	<i>vols per cent</i>		
July 1 9.45 p m			+	Very weak, Cannot stand up, apathetic, atonic, sleepy
10 05 "			+	Barking, howling, uneasy (aura convulsiva).
10 12 "	0 051	54	++	Completely passive, slight convulsions of the legs
10 50 "			++	<i>Strong convulsions of the neck muscles, strong opisthotonos, torsions of the whole body.</i>
11 00 "			++	Continuous convulsions.
11 12 "	0 060	55	++	Convulsions
11 27 "	200 gm of meat given			<i>Convulsions stopped immediately after ingestion and did not reappear Dog recovered</i>

Action of Glucose on Clinical Symptoms of Ketosis.

A small amount of glucose oxidized after ingestion of larger amounts of this sugar by the dogs which were in strong ketosis would suppress ketone body production (as it was shown in the preceding paper), and the animal, if in coma, would recover. When glucose action was over, symptoms of ketosis would reappear (acetone in breath, atony, semicomatose condition, Kussmaul breathing). The contrast between the period of strong ketosis and recovery was not always so striking as shown in Tables II or III because the dogs were very weak after long fasting. If there were any signs of the recovery to be noticed³ they could be expected at the time when acetone disappeared from the breath. That would happen with 20 to 30 gm of glucose between the 3rd to 9th hour, depending upon the quantity of sugar given. For the

³ The favorable influence of feeding carbohydrate and protein in phlorhizinized dogs was noticed first by Geelmuyden (7) and Lusk (8) The question has been studied since by several authors.

most part it corresponded closely to the lowest points of the curve of ketone bodies in the urine and in the blood. These would reappear in the breath when their curve in the blood and in the urine began distinctly to rise. And as ketone body elimination represents what happens with the metabolism of such dogs (as described in the previous paper), it was possible with a little practice by smelling the nose of such a dog and by judging its general appearance to follow its metabolism before any quantitative determinations were made.

Total and Extra Sugar Excretion in the Urine as Compared with Blood Sugar Curve.

To the same phlorhizinized dog, No. 12, 1 gm. of glucose per kilo of body weight was fed, and on another occasion 2.33 gm. of glucose per kilo. In both these experiments blood and urine were examined at the same time at frequent intervals. With 1 gm. per kilo (*cf.* Table II of Paper II), the blood sugar remained in this dog above the initial level for 5 hours, and afterwards dropped below it. The total sugar excretion of the urine followed exactly the blood sugar, and thus dropped to the initial level 6 hours after the ingestion of glucose. Extra sugar, however, was eliminated much longer, until the 16th hour after the feeding of glucose. In the second experiment with over 2 gm. of glucose per kilo (*cf.* Table IX, Paper II), the blood sugar was 9 hours above the initial level, and just so long was the total urine sugar higher than at the beginning of the experiment. Extra sugar excretion, however, lasted longer, persisting even while the blood sugar dropped below the initial level. This last fact may be explained by storage of glucose in the form of glycogen in the liver and eventually muscles, and by its slow after elimination as soon as a glucose "vacuum" again develops in the blood, and equilibrium is shifted from the glycogen side towards glucose.

In the preceding paper we have seen that the favorable influence of ingested glucose on nitrogen and ketone bodies stops when the total urine sugar excretion reaches the former level. From the above we notice that antispasmodic and ketolytic action last as long as the blood sugar value is above the preliminary level, and from the experiments on Dog 12 (Tables II and

IX, Paper II) we see that the blood sugar and total sugar excretion curves in the urine follow each other. It seems to be reasonable, therefore, to assume that the *favorable influence of glucose on phlorhizinized dogs, due to the sugar oxidation, is associated with its mass action, and lasts as long as the total sugar available for the body cells is above the preliminary level* (5 hours with 1 gm. per kilo, 9 hours with 2.33 gm. per kilo in a 13 to 15 kilo dog). It would be a case of protracted "plethora of carbohydrates" (Lusk (9)), longer than any one existing in physiological conditions. In studies of the respiratory metabolism of phlorhizinized dogs after ingestion of glucose (10), the author and those who kindly helped him were able to show an increase in respiratory quotient lasting a few (2 and more) hours after ingestion of glucose, and high enough to account for most if not all of the glucose which cannot be recovered in the urine as extra sugar. It will be left for future work to show whether or not increase (even though small) in respiratory quotient above the diabetic level may be observed as long as blood and total urine sugar are above the preliminary level.

Blood Sugar Curve of Phlorhizinized Dog as Compared with the Normal.

Comparison of the blood sugar curve of a phlorhizinized dog after ingestion of 30 gm. of glucose (Tables II and III; also Table IX, Paper II) with the curve of a normal dog after treatment with the same amount of glucose (Table IV) will allow us a few conclusions.

1. In this normal dog, the influence of 30 gm. of glucose on blood sugar was practically over in 5 hours. The increase of blood sugar in phlorhizinized dogs of similar size (Table III) lasted about twice as long.

2. In the normal animal, it reached the maximum at the end of the 2nd hour, in the phlorhizinized animal at the end of the 3rd hour. In absolute measure, the peak was lower in the phlorhizinized than in the normal animal, but its relative rise above the preliminary level was about the same.

3. After 9 to 10 hours the curve in phlorhizinized animals dropped 5 to 10 mg. below the preliminary curve. In our normal

dog it might have happened too, (perhaps during the 6th hour) if the blood samples could have been drawn long enough. This point has been shown by MacLean and de Wesselow (11) and Folin and Berglund (12) for normal human individuals. The following interpretation may be suggested for these differences and similarities in the blood sugar curves of fasting normal and phlorhizinized dogs.

Referring to (1) a protracted blood sugar value in a phlorhizinized animal in spite of the fact that there exists kidney leakage for sugar may mean a general hindrance in sugar utilization in the body cells in the sense of Nash and Benedict (13).

TABLE IV
Dog 11 Normal control fasting 2 days. Weight 12.4 kilos.

Time	Blood sugar	CO ₂ -combining power	Remarks
<i>1925</i>	<i>per cent</i>	<i>vols per cent</i>	
July 5			
11 55 a. m.	0.085	52	
12 00 "	30 gm glucose by stomach tube.		
1 00 p. m.	0.178	52	Sleeping.
2 00 "	0.206	51	Watchful.
3 00 "	0.165	52	"
4 00 "	0.105	51	"
5 00 "	0.091	52	"

As regards (2) the speed of increase of the curve and its relative height seem to be alike. If we remember, however, that a large fraction of sugar ingested is constantly dripping out through the renal artery into the urine (about 70 per cent during the whole time when the blood sugar curve was above the initial level), we see that the peak would be actually much higher in the phlorhizinized dog if, for instance, at the moment sugar was given the kidneys of the dog were suddenly injured, and sugar could not pass to the urine. As it is relatively just as high as in a normal animal (counting from the preliminary level), that also speaks for diminished ability to utilize sugar in the body cells as mentioned above.

As to (3), the drop in the blood sugar curve below the basal level may possibly be due to the insulin production from the islets going on excessively,⁴ after the need for it has stopped. Such an explanation implies the possibility of insulin secretion in phlorhizinized dogs, a situation which may be assumed with a large degree of certainty on the basis of the present work, because these dogs retain to a certain degree their sugar-oxidizing function (10, 15). Pancreas of phlorhizinized dogs is morphologically intact (16) and it contains insulin (17). No matter how much glucose we give to a phlorhizinized animal this sugar does not make the condition worse as it might in the ordinary human diabetic, but it always acts in a favorable way, and the favorable effect is directly proportional to the amount of glucose given. This shows that insulin secretion is in such animals adjustable to the needs. In short, the *endocrine function of the pancreas is retained in phlorhizinized dogs to a certain extent.*

CO₂-Combining Power in Blood of Phlorhizinized Dogs.

With progressing phlorhization the CO₂-combining power of the blood drops hand in hand with the increasing ketosis, but even in semicomatose dogs there have never been seen in this series of experiments such low figures as occur in human diabetic coma (Table V).

After ingestion of glucose by a normal fasting dog, the CO₂-combining power of the blood does not change (Table IV), as has been found already by Taistra (18). After ingestion of glucose by a phlorhizinized dog, the CO₂-combining power of the blood rises in the shape of a regular curve (Table III) which follows in a reciprocal way the ketone body excretion curve in the urine (Tables II and IX, Paper II).

At the same time, when the CO₂-combining power of the blood increases, ketone bodies in the blood decrease with the influx of sugar metabolites (Table VI). Table VI shows also that the decrease in ketone body excretion by the kidneys is not due to the retention of ketone bodies.

⁴ This explanation given by Foster (14) for hypoglycemias following glucose ingestion in normal individuals was suggested here to the author by Dr R. T. Woodyatt.

Keto-Antiketogenic Balance of the Dog.

When glucose is oxidized in a phlorhizinized dog, ketosis decreases or disappears with corresponding increase in CO₂-combining power of the blood which reaches its normal physio-

TABLE V

Progress of Acidosis in a Phlorhizinized Fasting Female Dog.

Weight 16.8 kilos

Time	CO ₂ -combining power of blood *	Concentration of ketone bodies in urine		General symptoms (Breath means acetone in breath)
		Acetone	Diabetic	
1926	vols per cent			
1-27 Fasting				Normal.
1-28 2nd day of fasting	64			Normal.
1-29 3rd day of fasting and phlorhizin	61			Normal.
1-30 2nd day of phlorhizin	55	+		Normal behavior. Breath?
1-31 3rd day of phlorhizin	51	++	+	Normal behavior. Breath +
2-1 4th day of phlorhizin	46	+++	++	Dog weaker. Breath ++
2-2 5th day of phlorhizin	43	+++	++	Dog very weak. Breath ++
2-3 6th day of phlorhizin	38	+++	++	Breath ++, dog very weak

* Blood drawn exactly at the same time of day from the same vein when the dog was quiet. Result is the average of two or three analyses checking closely.

logical value for a dog; the reverse happens when the influence of the oxidizing glucose stops. Brutto R.Q., corresponding in phlorhizinized dogs to the threshold of ketosis, is about 0.76 (10). Shaffer's (19) and Woodyatt's (20) formulæ applied to the metabolic mixture actually oxidized at the point when ketone

bodies disappear almost completely (determined in the calorimeter) hold true in such limits for the dog (10), as they have been shown to hold for man. Ingestion of fat by a phlorhizinized dog increases its ketosis (15). There is no need for an assumption that any other acids, besides ketone bodies, cause acidosis in these dogs,⁵ because the whole picture of acidosis fluctuates in them

TABLE VI
Dog 1. 9th Phlorhizin Day

Time	Blood			Urine.
	Sugar	CO ₂ -combining power	Ketone bodies	$\frac{D}{N}$ ratio
<i>1925</i>	<i>per cent</i>	<i>vols per cent</i>	<i>per cent</i>	
May 3				
3 45 a m.	0 055	38	0 038	2 97
4 03 "	30 gm glucose			
8 30 "	0 156	46	0 008	19 88

Dog 5 5th Phlorhizin Day.

Time.	Blood			Urine
	Sugar	CO ₂ -combining power	Ketone bodies	$\frac{D}{N}$
<i>1925</i>	<i>per cent</i>	<i>vols per cent</i>	<i>per cent</i>	
May 14				
1 22 a m	0 046	47	0 072	3 40
1 42 "	20 gm glucose.			
8 20 "	0 084	55	0 014	6 15
8 10 p.m.	0 048	43	0 054	3 23

closely with the rate of ketone body accumulation in the organism. Therefore the author feels justified in making the statement that the keto-antiketogenic balance of the dog is similar (if not identical) to that in the human.

⁵ Disappearance of ketone bodies in phlorhizinized dogs after renal injuries or during fever may also be explained on the uniform basis of increased sugar oxidation, an explanation which was already indicated for renal injuries by Allen (21).

SUMMARY.

Vital action of glucose in completely phlorhizinized fasting dogs concerns two syndromes either of which (or both) may cause death of the animal: (1) hypoglycemia with convulsions, and (2) ketosis with coma, both being visible exponents of the extremely low level of carbohydrate metabolism in the body cells.

1. In six out of the sixteen phlorhizinized dogs *convulsions* have been observed at the point when they apparently presented symptoms of the most severe form of diabetes. Glucose administered by mouth was successful in relieving convulsions (if the symptoms were not of too long standing) as long as blood sugar was above the preliminary level. Convulsions commonly returned as soon as the blood sugar dropped below the preliminary level. The blood sugar curve of a phlorhizinized dog as compared with a normal control is about twice as long and similarly high in spite of the large kidney leakage for glucose, both facts possibly indicating a general hindrance in glucose utilization. When the blood sugar curve drops, it usually descends below the preliminary level, a condition so often found in normal individuals. The total sugar curve in the urine in these animals corresponds to the blood sugar curve and the time when both are increased represents the time of the possible utilization of glucose.

2. The ingestion of sufficient quantity of glucose was followed by transitory disappearance of the clinical symptoms of *ketosis* and coma, when ketone bodies disappeared from the urine and breath. The CO_2 -combining power of the blood reflected the ketone body excretion in the urine, reaching its normal value for a dog when ketone bodies disappeared from the urine. It is concluded from this whole work that the keto-antiketogenic balance of the dog is similar to that in the human.

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THE DIFFUSION OF WATER INTO LECITHIN-COLLODION MEMBRANES.

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INTRODUCTION.

Loeb (1) has shown that collodion membranes treated with proteins show reversal of the sign of the charge on the membrane in the region of the isoelectric point of the protein used. That proteins alone may influence water transport through animal membranes is suggested by the experiments of Mudd (2). This author, by studying electroendosmosis through the pericardium, pleura, and mesentery, showed that these serous membranes also show a reversal of the sign of the charge on the membrane within a range of hydrogen ion concentration referable to the proteins present. This zone of charge reversal was practically independent of the amount of lipid contained in the tissue.

In general, the reversal of a membrane's charge seems to be due primarily to its amphoteric components. And the ease with which this reversal is occasioned is characteristic of changes of pH. Biologically, not only the proteins but also the phosphatides may play an important rôle in the determination of the electrokinetic state of an electroosmotic system. The isoelectric point of lecithin (from eggs) is given by Feinschmidt (3) as between pH 2 and 4. Levene (4) and his coworkers have resolved lecithin from eggs into two components, (1) lysolecithin, isoelectric zone pH 2.70 to 9.90, and (2) lysocephalin, isoelectric zone pH 5.5 to 7.5. The possibility may be considered that with protein predomination in the surface of the cells of the membranes used by Mudd, the effect of an amphoteric lipid component may be obscured. And further, in a simpler system such lipids may present charged

surfaces through oriented interfaces or an adsorption mechanism (Mudd (2)).

The preceding remarks make the study of the influence of hydrogen ion concentration on the diffusion of water through a simpler ampholytic lipid system biologically of interest. And such experiments may throw further light on the possible rôle played by the phosphatides in determining water transport. It is the purpose of this communication to present such a series of experiments where a lecithin-collodion membrane was employed, and the influence of pH on water transport through membranes of this type containing solutions of a bivalent anion and a bivalent cation was studied.

Method of Membrane Manufacture.

The method of preparation and some properties of these lecithin-collodion membranes have been previously described in detail (5). It is important, however, to reemphasize here, that membranes of low drying times, *e g.* of 2 minutes, may not be used in experiments of a similar type. Membranes of low drying times have a lower and varying permeability. Those of the later drying times on the other hand show relatively similar osmotic phenomena. The membranes used in the experiments to be reported contained a calculated 50 per cent by weight of lecithin and were allowed to dry for 5 minutes. These membranes are delicately opalescent. Membranes of minimum drying time (previously reported) are clear. This suggested the possibility that, with continued drying, a transition from

- | | | |
|--|---|--------|
| (1) Guncotton. }
Lecithin. }
Ether. } solution. —————→
Alcohol }
(Water) } | (2) (1 min , 30 sec drying time, mem-
brane clear.)
Guncotton. }
Lecithin. } solution.
Ether }
Alcohol. } in traces.
Water. } | —————→ |
| (3) (4 min. drying time, opalescent membrane.)
Guncotton. }
Lecithin. } suspension of lecithin
Very faint traces of solvents. | | |

may have occurred. Microscopic studies of stained and unstained membranes showed no easily determined morphology. The

lecithin used was Merck's lecithin from eggs. The zone of maximum flocculation was pH 2 to 4. The experiments were done in 1921.

EXPERIMENTAL.

The method used was identical with that of Loeb (1). His curves for ordinary (untreated) collodion membranes were also obtained in the authors' control series. The method in brief is as follows: The mouth of Erlenmeyer-shaped collodion or lecithin-

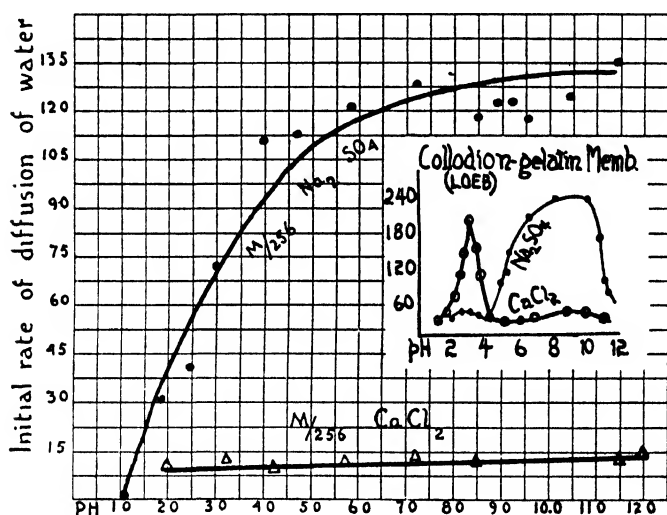


FIG 1. Lecithin-collodion membranes. There is no difference between this curve and control collodion membranes.

collodion sacs is stoppered with 1-hole rubber stoppers by means of rubber bands. Into the stopper is inserted a 2 mm. glass tube to be used as a manometer. The initial rate of diffusion of water into the bags was measured when each contained the same concentration of salts made up in varying hydrogen ion concentrations with HCl, and immersed in 350 cc. of water of the same pH at room temperature. From these values a curve of diffusion rate can be plotted against pH. Further details should be sought in the papers of Loeb and of the authors.

RESULTS AND DISCUSSION.

Through the range studied (Fig. 1), pH 2 to 12, there is no difference between curves obtained from Na_2SO_4 and CaCl_2 in collodion sacs and lecithin-collodion sacs. The curves obtained from pure collodion are, within the limits of experimental error, identical with that of Fig. 1. The small curve is typical of gelatin-treated membranes (Loeb).

Through the biologically important range of pH then, the influence of lecithin on water transport through lecithin-collodion membranes is negligible. But the possibility still exists, that at much higher concentrations of hydrogen ion points of inflection may be met with. These were not studied. One may explain the negative results through the low (pH 2 to 4) isoelectric range of lecithin; or one may hypothecate that no molecule orientation of charged surface may be present to influence the charge of the membrane (see Freundlich (6)). In this case the surface presented to the ions would be the membrane obtained from collodion, with the lecithin surfaces underlying the collodion surface and not in contact with the solutions

CONCLUSIONS.

The presence of 50 per cent of lecithin in a membrane prepared from a collodion-lecithin solution does not appreciably alter the diffusion of water into these membranes through a range of pH 2 to 12. The nature of the state of lecithin in the membranes and the possible biological significance of membrane phosphatides is briefly discussed in relation to water transport.

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LACTIC ACID FORMATION IN MUSCLE EXTRACT.

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Several attempts had been made to show that an enzyme or group of enzymes capable of converting carbohydrate into lactic acid could be extracted from muscle tissue before it was successfully demonstrated by Meyerhof in 1926. In 1902, Stoklasa (14) and coworkers reported the separation of a ferment from extracts of lung and muscle which was said to convert added glucose into lactic acid, and in 1910, Ransom (12) claimed to have demonstrated the production of lactic acid from glucose by muscle plasma of the frog and fowl. Fletcher (6) could not confirm the work of either, and ascribed their results to bacterial action.

In 1912, Embden, Kalberlah, and Engel (3) determined the lactic acid formed spontaneously in muscle press juice and found that there was a definite increase after 1 to 3 hours incubation at 40°C. even though the lactic acid content of the juice was relatively high by the time its preparation at 0°C. had been completed. The addition of glucose, glycogen, *D*-alanine, and inositol did not affect the total amount of lactic acid formed, hence they concluded that the precursor of lactic acid was none of these. Embden suggested that the unknown precursor be called lactacidogen, and later (4) obtained data which allowed the conclusion to be drawn that this substance was hexose phosphate and that it was similar if not identical to the hexose diphosphate formed by yeast juice during incomplete fermentation of sugar (9). The spontaneous formation of lactic acid in the muscle press juice was accompanied by an increase in inorganic phosphate which corresponded to a ratio of 1 molecule of lactic acid to 1 molecule of phosphoric acid. An osazone which had the same melting point as the osazone of yeast hexose phosphate was obtained from

fresh muscle, hence it seemed likely that the formation of lactic acid was due to the cleavage of hexose phosphate in the muscle juice.

Laquer and Griebel (10) in investigating the effect of adding carbohydrate to frog muscle found that glycogen, yeast hexose phosphate, and starch greatly increased the production of lactic acid during 3 to 5 hours incubation at 28°C. α -Glucose was found to be more effective than β -glucose, but both were less effective than glycogen. No phosphate determinations were made.

In 1924 Embden and Hayman (5) studied the behavior of the inorganic phosphate in freshly prepared dog and rabbit muscle press juice and found that in the unmodified juice the phosphate increased about 50 to 100 per cent during 2 hours incubation at 40 to 45°C. If however sodium fluoride were added, the inorganic phosphate decreased and if both fluoride and glycogen were added the inorganic phosphate decreased to a mere trace. An osazone which had a melting point of 150° (comparable to the osazone of yeast hexose phosphate), and which had a phosphorus content that agreed with the theoretical requirement, was isolated from the incubated juice to which sodium fluoride and glycogen had been added. No data were given for any of the other substances (glycogen, glucose, and lactic acid), which presumably participate in the reaction involving the synthesis of hexose phosphate, and since the amount of inorganic phosphate which disappeared was greatly increased by the addition of glycogen, but not affected by the addition of glucose or maltose, it seemed that the ferment action involved the esterification of the sugar at the moment of the splitting of the glycogen molecule. The function of the fluoride seemed to be that of an esterase inhibitor, thus allowing the synthetic action to become more apparent.

Beattie and Milroy (1) determined the changes in phosphate, glycogen, and lactic acid which occurred in hash made from the muscles of dogs, cats, and rabbits. This work appears to be the first attempt by means of simultaneous determinations to correlate the interrelated changes which occur among the three metabolites. They have shown that under the influence of sodium fluoride a much greater amount of glycogen is lost than is accounted for by the amount of lactic acid formed plus the amount

of inorganic phosphate decrease. Unfortunately their results cannot be interpreted as expressing any definite relationship between the loss of carbohydrate and the formation of lactic acid and hexose phosphate since they made no determinations of free or total sugar in the hash at the end of the incubation periods. The amylolytic action of the muscle hash on the glycogen would conceivably account for the greater part of the glycogen loss.

The most recent contributions to the knowledge of the enzymatic formation of lactic acid in muscle hash or muscle extract are those of Meyerhof (11). In 1926 he announced the demonstration of a ferment in extracts obtained from frog and rabbit muscle that was capable of producing lactic acid from carbohydrate, particularly from glycogen and starch. He pointed out that the production of lactic acid from hexose phosphate by this ferment was slower than the production of lactic acid from either glycogen or starch and considered this to be evidence that hexose phosphate could not be looked upon simply as an intermediary product between glycogen and lactic acid in the sense in which it had been considered by Embden. Later (August, 1926) he found that the extract maintained its ability to split hexose phosphate after it had lost its activity toward polysaccharides through deterioration. The ability of extract made from frozen rabbit muscle to convert glucose and fructose into lactic acid was lost very quickly but could be restored by a coferment prepared from autolyzed yeast. This coferment was obtained by precipitating yeast autolysate with 50 per cent alcohol and purifying the precipitate by resolution in water and reprecipitation with alcohol. When a small quantity of this coferment was added to muscle extract which had lost its activity toward hexoses the activity was not only restored but even increased over its original value. Fructose was much more readily attacked than glucose under these conditions. His later findings are therefore more in harmony with Embden's theory that hexose phosphate is an intermediary between glucose or glycogen and lactic acid. Meyerhof considers that in order to explain all his results it is necessary to assume the existence of a labile hexose monophosphate as well as a stable diphosphate and that the diphosphate accumulates during the first period of the reaction in approximately equimolecular ratio to the monophosphate hydrolyzed. During the second

period of the reaction, if sugar is absent, the diphosphate is slowly broken down to free phosphate and lactic acid.

The work reported in this paper represents an endeavor to obtain data which would permit assigning more definite stoichiometric relationships between carbohydrate, lactic acid, and inorganic phosphate when these substances undergo changes in muscle extract. In order to make a comparison between the carbohydrate loss and lactic acid gain and at the same time correlate the changes which were taking place in the inorganic phosphate we made simultaneous determinations of the free sugar, total carbohydrate after acid hydrolysis, lactic acid, and inorganic phosphate

Some preliminary experiments made with muscle hash, with extracts, residues of tissue from which the extracts were made, and with mixtures of extracts and residues, by the same general procedure described below showed the following facts. The addition of glycogen greatly increased the amount of lactic acid formed while the addition of glucose had little if any effect. When sodium bicarbonate buffer was used the decrease in total sugar approximately corresponded with the increase of lactic acid, but with phosphate buffer there was a greater loss of total carbohydrate than could be accounted for by the lactic acid formed. This indicated that the phosphate was responsible for the disappearance of a part of the carbohydrate, and in subsequent experiments determinations of the inorganic phosphate were made. These later experiments were limited to muscle extracts because the extract represented a simpler system, and the unavoidable errors of sampling a tissue hash were eliminated. The results obtained from extract were much more uniform than those obtained from tissue

The muscle extracts were prepared by mixing 3 volumes of distilled water with 1 part (by weight) of ground rabbit muscle. The muscle was taken immediately after the killing (by bleeding) of the animal which had been anesthetized with amytal administered intraperitoneally (75 mg. per kilo of body weight). When anesthesia was complete the hind legs were put into a mixture of ice and water and allowed to become cool (about 15°C.) before the animal was killed. The hind legs were skinned as quickly as possible after death, and the muscles removed and ground

in an ice-cold meat chopper. This ground tissue was weighed and put into a measured quantity of distilled water previously cooled to 0°C. At the end of $\frac{1}{2}$ hour at 0°C. the mixture was strained through unbleached muslin and the tissue residues squeezed out by twisting the cloth around it.

The extract obtained in this manner contained a few blood cells, was pink-colored, and somewhat turbid. Further attempts to purify it by centrifugalizing were not made since any delay resulted in considerable loss of its lactic acid-forming ability.

One experiment was performed with tissue extract obtained from an animal which had been killed instantly by a blow on the head without anesthesia. The results obtained were similar to those obtained from anesthetized animals except that the initial lactic acid content of the muscle and extract was much higher. It seemed desirable therefore to use amytal anesthesia so that the nerve impulses sent to the muscles during the death struggles would be blocked and a low initial lactic acid content thereby obtained. The grinding of the muscle did not cause the marked lactic acid formation which was produced by the convulsive movements.

Since a carbohydrate deficit appeared in the experiments performed with phosphate buffer, while all the carbohydrate was accounted for in those in which sodium bicarbonate was used, it seemed highly desirable to endeavor to determine the relationship of this phenomenon to the phosphate. If the carbohydrate were converted into hexose phosphate it would conceivably fail to reappear when the total carbohydrate is determined after acid hydrolysis. Furth and Marian (8) have shown that when hexose diphosphate (yeast) is hydrolyzed by 2½ per cent HCl for 3 hours only one-third of the theoretical amount of the hexose is obtained when it is determined by copper reduction. The carbohydrate which disappeared in our experiments, when an excess of phosphate was present, presumably could not be recovered completely as sugar but would be accounted for by a corresponding disappearance of inorganic phosphate.

EXPERIMENTAL.

50 cc. portions of extract were mixed with buffer and water or buffer and glycogen solution as follows:

Flask No	20 cc buffer	4 per cent glycogen solution	Distilled water
		cc	cc
1	B		10
2	"	10	
3	P		10
4	"	10	
5	F		10
6	"	10	

The buffer solutions were made as follows:

	M
B. NaHCO_3	0 16
NaCl	0 20
P. NaHCO_3	0 12
K_2HPO_4	0 04
NaCl	0 20
F Same as P plus NaF	0 1

Immediately after mixing, a sample (15 cc.) was withdrawn from each and delivered into 20 cc. of 6 per cent HCl. This sample served as the control on the initial content of carbohydrate, lactic acid, and inorganic phosphate. The remainders of the mixtures were incubated in a water bath at 24.5°C. 15 cc. samples were withdrawn at 0.5, 1, 2, and 4 hour intervals, and treated in the same manner as the control samples.

Each sample was prepared for analysis as follows: The proteins were precipitated after acidification with HCl by the addition of 25 cc. of 5 per cent HgCl_2 according to the Schenk process. The tubes containing the precipitated protein mixture were allowed to stand overnight and then filtered. 10 cc. portions of each filtrate were diluted with an equal volume of 2 per cent HCl and hydrolyzed in a boiling water bath for 3 hours. The mercury was removed by H_2S and the H_2S removed by aeration. Total sugar determinations were made on the filtrates obtained from these hydrolyzed samples. The remainders of the original filtrates were freed from mercury in the same manner as the hydrolyzed portions, and determinations made for free sugar, lactic acid, and inorganic phosphate.

Sugar was determined by the Shaffer-Hartmann method (13),

lactic acid by the Friedemann, Cotonio, and Shaffer procedure (7), and phosphate by Briggs' modification of the Bell-Doisy method (2).

The effect of the presence of mercury in the hydrolyzing process for total sugar was ascertained by adding an amount of 5 per cent HgCl_2 solution comparable to that used in the experiments to a known solution of glycogen. This test was made for both pure glycogen and glycogen plus a small amount of protein material. The HgCl_2 had no effect on the results provided it were removed before sugar determinations were made.

All filtrates were neutralized with 55 per cent NaOH to approximately pH 5.0 before any of the determinations were made. The addition of the alkali increased the volume and introduced an error in the absolute values obtained which was in the opposite direction from the error caused by the concentration of the filtrates during aeration. No corrections have been applied for these errors because each sample was subject to the same treatment, hence the relative values are not affected. A consideration of other sources of error including the limitation of the analytical method indicates that they are too small (2 to 5 per cent) to affect the interpretation of the results obtained. The largest error is in the determination of free sugar in the samples which contained only a small amount of sugar. Mercuric chloride in acid solution does not remove all of the interfering reducing bodies, hence these values are high. This error does not materially affect the free sugar determinations where a relatively large quantity of sugar was present.

The experimental data obtained are given in Table I. Flasks 1 to 4 contained extract from one animal and Flasks 5 and 6 from another. A control on Flasks 5 and 6 containing glycogen but no fluoride was very similar to No. 4, and is not included because it furnishes no additional information.

DISCUSSION

The interpretation of the data is concerned chiefly with the balance that may exist between the three metabolites studied, and is based on the assumption that the reaction involved proceeds in the following manner.

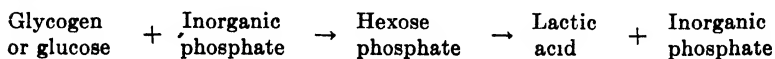


TABLE I.

Flask No.	Incubation time	Millimols of substances found in 15 cc. of incubated mixture.			
		Lactic acid	H ₂ PO ₄	Free sugar	Total sugar.
Control.	<i>Hrs.</i>				
	0	0 13	0 12	0.021	0.089
	0 5	0.17	0 12	0.017	0.078
	1	0 18	0.14	0 015	0.067
	2	0 18	0 14	0 015	0.078
	4	0 18	0.14	0 020	0 078
Glycogen added.	0	0 14	0 12	0 023	0 54
	0 5	0 21	0 11	0.032	0 46
	1	0 23	0 08	0 067	0 45
	2	0 27	0 06	0 087	0.44
	4	0 28	0.05	0 101	0 42
Phosphate added	0	0 14	0 24	0 021	0 09
	0 5	0 17	0 23	0 017	0 08
	1	0 18	0 23	0 015	0 08
	2	0 18	0 25	0 017	0 07
	4	0 19	0 25	0 021	0 08
Glycogen and phosphate added.	0	0 15	0 23	0 021	0 53
	0 5	0 23	0 22	0 045	0 47
	1	0 275	0 17	0 082	0 44
	2	0 285	0 14	0 112	0 41
	4	0 30	0 12	0 132	0 35
Phosphate and fluoride added.	0	0 19	0 23	0 028	0.11
	0 5	0 18	0 23	0 017	0 09
	1	0 18	0 22	0 017	0 09
	2	0 18	0 22	0 017	0 08
	4	0 18	0 22	0 028	0 09
Glycogen, phosphate, and fluoride added.	0	0 18	0 22	0 033	0.45
	0 5	0 18	0 06	0 08	0 35
	1	0 19	Trace.	0.10	0 32
	2	0 19	0	0 12	0 31
	4	0 19	0	0 12	0 30

If glycogen (or glucose) passes through a phosphate complex in being converted into lactic acid by muscle enzymes, and if no other reactions involving carbohydrate, phosphate, and lactic acid

occur simultaneously, it should be possible to balance the molar equivalents of these substances according to the following equation.

$$\begin{array}{rcccl} \text{Loss of total} & & \text{Loss of inorganic} & & \text{Gain of} \\ \text{carbohydrate} & = & \text{phosphate by forma-} & + & \text{lactic} \\ & & \text{tion of hexose phosphate} & & \text{acid} \end{array}$$

Each mol of glucose lost is equivalent to 2 mols of lactic acid formed, or may require either 1 or 2 mols of phosphoric acid for esterification to give a monophosphate or a diphosphate. The values obtained for total carbohydrate must be used, since the free sugar is included in this figure. Furthermore, the free sugar represents merely that portion of the glycogen which was converted into reducing sugar (calculated as glucose) by the amyolytic action of the muscle extract and escaped conversion into lactic acid.

The total carbohydrate (calculated also as glucose) represents the copper reduction value obtained after hydrolysis by acid of all polysaccharides present. This value is accurate for the glycogen and free sugar present at the beginning of the incubation periods, but after the incubation is in progress the results become less certain because the amount of reducing sugar obtained from the hydrolysis of the carbohydrate involved in the phosphate complex is not accurately known. Since only one-third of the glucose in the phosphate complex of yeast is recovered by acid hydrolysis (8), it seems likely that hydrolysis of the phosphate complex of muscle may yield only a part of its carbohydrate as reducing sugar. This possible source of sugar has been ignored in the trial calculations of the relationship represented by the equation given above.

The values for the substances studied are expressed in millimols to facilitate comparisons. The data given in Table I show the total amounts found in the samples analyzed for the different time intervals, while Table II and Fig. 1 summarize the changes which occurred during the entire 4 hour period.

Flasks 1 and 2 contained only the phosphate which was present in the muscle extract, and Nos. 3 to 6 had an amount of phosphate added which approximately doubled that originally present.

The changes which occurred in Flasks 1, 3, and 5 are so small that it is doubtful whether the figures can be applied to the hy-

pothetical equation. It is noteworthy that the increase of lactic acid, which occurred in Flasks 1 and 3, was prevented by the fluoride in No. 5. The changes which occurred in Flasks 2, 4, and 6, to which glycogen was added, have sufficient magnitude to justify an attempt to fit them into the equation. There was considerable disappearance of total carbohydrate in them, which is accounted for only in part by the lactic acid formed. This is particularly noticeable in Flask 6, in which the formation of lactic acid was almost completely inhibited by the fluoride. Here, the loss of carbohydrate occurred and the decrease in inorganic phosphate is more marked than in the absence of fluoride.

Table III gives a summary of the calculation. The gain of

TABLE II
Total Amount of Change in Millimols for 4 Hour Period

Flask No	Substances added	Lactic acid	H ₃ PO ₄	Free sugar	Total sugar
1	None	+0 05	+0 02	0	-0 01
2	Glycogen	+0 14	-0 07	+0 08	-0 12
3	Phosphate	+0 05	+0 01	0	-0 01
4	Glycogen and phosphate.	+0 15	-0 11	+0 11	-0 18
5	Phosphate and fluoride.	-0 01	-0 01	0	-0 02
6	Glycogen, phosphate, and fluoride	+0 01	-0 22	+0 09	-0 15

+ gain, -- loss.

lactic acid expressed as equivalent millimols of glucose (mm lactic acid \div 2) is subtracted from the loss of total sugar and gives the amount of carbohydrate presumably present as a phosphate complex. This value can be compared with the decrease of inorganic phosphate.

The deficit of sugar is in fairly close molecular agreement with the loss of phosphate, and the calculation is therefore consistent with the idea that the carbohydrate may have been present in a complex, equivalent to hexose monophosphate. In order to justify this agreement it is necessary to assume that sugar was not liberated from this complex during acid hydrolysis, for if such had occurred, and the liberation had been quantitative there would have been no carbohydrate deficit. The loss of phosphate,

however, would have occurred since phosphate determinations were made on the unhydrolyzed samples. Satisfactory phosphate determinations were not obtained on hydrolyzed material because so much phosphate was liberated by hydrolysis that it seemed likely that there was some source of phosphate other than that associated with the carbohydrate changes.

It is not possible to decide the question involved with the data available at present, but we can tentatively interpret the results

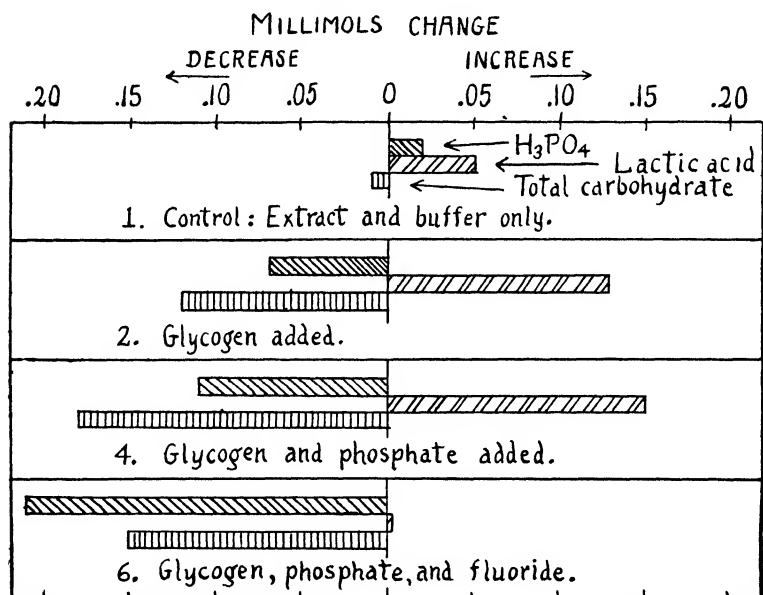


FIG. 1.

as supporting the hypothesis that glycogen is converted into a phosphate complex, the composition of which corresponds to hexose mono- or diphosphate. The enzymatic hydrolysis of the complex yields 2 molecules of lactic acid and 1 or 2 of inorganic phosphate. The rather remarkable effect of fluoride in preventing the hydrolysis of the complex by the enzyme should be the means of producing an accumulation which may lead to its isolation. Efforts to do this are being undertaken.

SUMMARY.

1. Simultaneous determinations of lactic acid, inorganic phosphate, free sugar, and total carbohydrate were made on incubating mixtures of fresh muscle extract in a buffered solution.

2. The addition of glycogen to the extract caused a marked increase in lactic acid formation, and usually a decrease in the inorganic phosphate. The amount of free sugar increased as a result of hydrolysis of the glycogen by the amylolytic ferment of the muscle.

3. The loss of carbohydrate from mixtures to which glycogen was added was about twice as great as the amount of lactic acid formed.

4. The addition of phosphate in sufficient quantity to double the amount naturally present in the extract caused only a slight

TABLE III

Flask No	2	4	6
Substances added	Glycogen	Glycogen and PO_4	Glycogen, PO_4 , and NaF
Decrease of total sugar, $\text{m}\mu$	0 12	0 18	0 15
Gain of lactic acid - 2	0 07	0 075	0 005
Differences	0 05	0 105	0 145
Decrease of H_3PO_4	0 07	0 11	0 22

increase in the amount of lactic acid formed and carbohydrate used, provided glycogen also were added. If no glycogen were added no noteworthy changes took place.

5. The addition of fluoride prevented the formation of lactic acid and augmented the decrease in inorganic phosphate, but did not prevent the decrease in total carbohydrate.

6. The difference between the carbohydrate loss and lactic acid formed was approximately equal to the loss of phosphate. This relationship suggests the formation of a carbohydrate-phosphate complex.

7. Our results confirm those obtained by Embden and by Meyerhof, and show in addition that there is a simultaneous decrease in carbohydrate when the free phosphate decreases and lactic acid is formed.

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THE EFFECT OF THE ADMINISTRATION OF PARATHYROID EXTRACT ON NORMAL CALVES.*

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The work herein described was undertaken as a part of the study of the results of the feeding of certain restricted rations to dairy calves which apparently produced disturbances in their calcium-regulating mechanism (1).

The effect of the administration of parathyroid extract to various animals has been studied recently by Hansen (2), Collip (3-6), Macleod (7), Hjort (8), and Greenwald (9, 10), and their coworkers who found a wide variation in the resistance of various species of animals. Macleod (7) says that herbivores are particularly resistant to it; Collip (3) says that rabbits are relatively immune; while Greenwald (10) states that cats, rabbits, and rats are much less sensitive than are dogs. Greenwald and Gross (9) have shown that the administration of parathyroid hormone to dogs not only causes a rise in blood calcium but also a marked increase in the excretion of calcium and phosphorus. In view of the extensive experiments carried on with dairy cattle for the purpose of studying their calcium and phosphorus metabolism this discovery of a definite substance capable of influencing the excretion of these substances became of great interest. The apparently wide variation in susceptibility of various animals to the action of this hormone made unsafe the application to dairy animals of the results obtained with dogs. Consequently it seemed advisable to study its effect directly on calves.

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Four experiments were conducted using in all ten animals. In two experiments on three and four animals respectively, the calcium and phosphorus balances were determined. The results are summarized in Tables I and II

In two of the experiments definite overdosage symptoms were observed. Calf P₂ in Experiment 2, receiving 200 units¹ in 50 unit doses, became increasingly drowsy as the treatment progressed but revived as soon as they ceased. In Experiment 3 two of the three animals became bloated and all were apathetic. There was no indication of diarrhea but in all cases the urine was increased in volume and passed more frequently than usual. There was of course no vomiting as there is with dogs. Calf P₅ which was killed by the administration of 1300 units in 100 unit doses became drowsy and weak but showed no other symptoms. The urine and feces were not collected from this animal.

Blood samples were taken from the jugular vein and analyzed for calcium and phosphorus by the methods used in previous work (11). Some blood sugar determinations were also made but the values were all normal.

DISCUSSION.

Blood.—Two points are of interest; *viz.*, the actual effect of parathyroid extract on the blood picture of the calves, and the relative magnitude of the results as compared with those produced by the extract on other animals.

Qualitatively the results are the same; *i.e.*, a marked rise in the blood calcium and a tendency for the inorganic phosphorus to decrease.

The blood calcium curve for the one animal that died resembles in form the typical one as described by Collip ((5), p. 504) while the others correspond to it except that they return to normal after the cessation of the injections. The maximum is at about the 18 mg. level instead of the 20 mg. level.

The effect of pyramiding doses appears to be less pronounced with our calves than it was with Collip's dogs. The two calves receiving a total of 200 units in eight and four injections showed

¹ For the first work, extract prepared in this laboratory by the original method of Collip was used. For all later work parathormone Lilly was used. We are indebted to Eli Lilly and Company for a portion of this material.

TABLE I.
Effect of Parathyroid Extract on Blood Calcium of Calves.

Experiment No.	Animal No	Weight.	No. of doses.	Units per dose	Total units.
		<i>kg.</i>			
2	P ₁	39	8	25	200
	P ₂	50	4	50	200
	P ₃	43	1	200	200
	P ₄	34	13	100	1300
3	P ₁	102	1	400	400
	P ₂	82	9	100	900
	C63	125	2	400	800
4	P22	85	1	340	340
	P30	75	1	300	300
	P36	68	1	540	540
	P66	68	1	540	540

TABLE II
Average Daily Values for Ca and P Intake and Excretion in Gm.

Experiment No.	Animal No	Calcium.				Phosphorus			
		Intake	Urine	Feces.	Total excreted.	Intake.	Urine.	Feces.	Total excreted
3	P ₁	A 27 23	0 08	13 83	13 91	9 62	0 36	2 57	2 93
		B 24 04	0 18	14 55	14.73	9 39	3 31	2 37	5 68
	P ₂	A 18 82	0 12	7 15	7 27	8 22	0 10	1 47	1 57
		B 16 51	0 21	7 62	7 83	7 84	2 78	1 64	4 42
	C63	A 37 91	0 35	23 30	23 65	11 72	0 05	4 10	4 15
		B 37 11	0 17	23 71	23 88	11 70	1 89	4 12	6 01
4	P22	A 17 41	0 05	12 00	12 05	6 61	1 12	2 96	4 08
		B 17 26	0 13	12 00	12.14	6.81	1 52	3 08	4 80
	P30	A 10 13	0 08	5 85	5 93	5 70	2 47	1 71	4 18
		B 10 03	0 40	7 48*	7.88	6 03	3 99	1 31	5 30
	P36	A 9 25	0 06	6 60	6 67	5 06	2 07	1 35	3 42
		B 9.14	0 20	6 60	6 80	5 27	3.16	1.04	4 20
	C66	A 17 34	0 05	12 06	12.11	6 35	1 53	1 84	3 37
		B 17 20	0 06	11 69	11.75	6 55	3 02	1 46	4 48 .

A values are averages for 6 days and 7 days preceding injection in Experiments 3 and 4 respectively.

B values are averages for 4 days after injection.

* Due to the passage of an unusually large quantity of material on 1 day.

no appreciable effects, although the one receiving the same total in one dose showed a distinct rise in blood calcium. Calf C63 receiving only two 400 unit injections suffered an increased calcium of only 4.2 mg. as compared with a rise of 6.3 mg. in Calf P₁ which received but one dose of 400 units. Our Calf P₂ which received nine injections of 100 units each, to total 10.8 units per kilo of body weight, offers a definite indication of comparative resistance. A dog receiving a corresponding amount of extract would, judging from Collip's figures, have died or at least shown marked overdosage symptoms. This calf while showing some disturbance was not, apparently, particularly uncomfortable and promptly returned to normal. Likewise, the one calf which did die lived long enough to receive 37.7 units per kilo, whereas Collip's dogs succumbed after the injection of less than 20. The absence in cattle of that outstanding canine reaction, the ability to vomit, makes the accurate comparison of symptoms difficult, but it is evident that calves do show a greater resistance to the overdosage effects than do dogs.

Calcium and Phosphorus Excretion—In every case there was observed an increase in the quantity of phosphorus excreted in the urine, and in all but one case the same was true for calcium. The fecal excretion of these elements revealed no such consistency. The values for the daily excretion of calcium showed that any fluctuations in fecal output due to treatment were so small that they were quite concealed by the normal day to day variations, although the 4 day averages after injection show a slight increase in most cases. The fecal phosphorus, on the other hand, shows a decrease or no change in most cases. In this respect, our results differ from those of Greenwald who found with dogs an increased excretion of calcium and phosphorus in the feces as well as in the urine. In only one of his experiments is it possible to calculate the balance but in this one, his long time experiment (No. 6b), the animal was apparently in negative balance with respect to both calcium and phosphorus throughout the period of observation even when not receiving extract. In our last experiment two animals were placed on a high calcium and two on a low calcium ration, though one which still held them in positive balance. This was without any apparent effect on the fecal excretion, so we are forced to conclude that either we are dealing with a phenomenon

associated with the different types of animals employed or that our reduction of intake was not sufficiently drastic.

Consideration of the quantities of material which was excreted shows that the welfare of the animal may usually be endangered only through the excessive loss of phosphorus, the increased loss of calcium being too small to be of importance. Greenwald (10) suggests as one explanation for the greater resistance of some animals to the action of parathyroid extract that "their mechanisms for the excretion of calcium function more readily and they, therefore, never develop a dangerous hypercalcemia." We have shown that calves are apparently more resistant than dogs, yet it appears that the increase in the amount of calcium excreted is insignificant. Collip (6) has shown that hypercalcemia produced by the injection of calcium salts alone is not injurious to dogs whereas the simultaneous injection of calcium and phosphate solutions produced typical parathyroid overdosage symptoms. The inference from this is that in dogs an increase in the concentration of both calcium and phosphate ions is produced by the injection of parathyroid extract and that this gives rise to the symptoms. Greenwald's observation that the excretion of both calcium and phosphorus follows such injection tends to support this assumption that both are directly or indirectly concerned in the hormone action. In calves, on the other hand, ionic calcium is apparently not liberated to the same extent (or is immediately recombined) with the resulting appearance of resistance on the part of the animal. Until more data are available, any speculation on the mechanism of the process is futile.

Those of our animals which showed no overdosage symptoms generally showed smaller increase in the elements excreted than did those animals showing symptoms.

SUMMARY.

The injection of a potent extract of parathyroid glands into calves produced a marked increase in blood calcium. If sufficient extract were administered, a maximum of 18 mg. per 100 cc. was reached in about 30 hours after the first injection. Increasing the dose of extract failed to raise the blood calcium above this value.

There was a decrease in inorganic phosphorus of the blood.

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The visible symptoms of overdosage were drowsiness and bloating. Continued injection resulted in death.

Calves apparently are less sensitive than dogs.

There is a marked increase in the phosphorus and calcium excreted in the urine but no consistent change in the fecal excretion of these elements. While the urinary excretion of calcium is relatively decidedly increased, the actual increase is insignificant in the metabolism of the animal. It is suggested that the relative resistance on the part of calves as compared with dogs is due to the smaller amount of ionic calcium produced by the hormone action, or by the ability of the animal to recombine it.

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ON THE EXISTENCE OF TWO ACTIVE FACTORS IN THE VITAMIN B COMPLEX.*

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Critical reviews of the literature by other investigators (4, 6) have presented several reasons for dissenting from the view that vitamin B is a single substance possessing both antineuritic and growth-promoting properties. One of the reasons mentioned was an indication in a number of experiments of a lack of correlation between the antineuritic and the growth-promoting actions of green vegetables, roots, and tubers as compared with these same properties of the grains. The experiments were not strictly comparable, however, since the tests were not carried out upon the same samples of materials.

A further reason was advanced by Funk and Dubin (1) in a preliminary paper which reported a partial separation of a yeast extract into an antineuritic fraction and a fraction exerting a specific growth-promoting influence upon microorganisms. The latter fraction was said to be necessary, in addition to the antineuritic fraction, for the growth of rats. No experimental data were submitted and we have not been able to find a more complete report.

Three extremely interesting papers have appeared during the progress of the present investigation. Goldberger *et al.* (2, 3) in studying the relation of their factor P-P to vitamin B have shown that, when rats were fed a vitamin B-free diet supplemented by 8 or 10 per cent of autoclaved yeast and 5 per cent of an alcoholic extract of maize meal, they made significant growth. However, when as much as 40 per cent of either supplement alone was added to the basal diet, the rats failed to grow.

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In the other paper Smith and Hendrick (7) have reported that diets containing large proportions of rolled oats have been noticeably improved for the growth of rats by the addition of 5 per cent of dry brewers' yeast which had been autoclaved for 6 hours at 15 pounds pressure. They also reported that 1 to 2 mg. daily of Seidell's picrate as the sole source of vitamin B did not enable rats to grow unless it was further fortified with the autoclaved yeast. These results were attributed to some relatively heat-stable factor other than the antineuritic vitamin

The objects of the experiments described herewith were: (1) to determine the relative antineuritic and growth-promoting values of the same samples of plant materials; (2) to attempt a separation of extracts from these materials into fractions that might possess either the antineuritic or the growth-promoting action alone.

EXPERIMENTAL.

Methods.

Plant Materials Used

Seeds of the Mammoth Yellow variety of soy beans and the Early Speckled variety of velvet beans were tested. The seeds were crushed, dried in an air oven at about 45°C., and ground until they would pass through a 40 mesh sieve. Leaves of the same variety of velvet beans and leaves of the Dwarf Essex variety of rape were used in the tests on leafy materials. The leaves were harvested and spread on the floor of an attic room to dry. The air-dry material was then dried overnight in an air oven at 40-45°C. and ground to a powder.

Preparation of the Extracts.

Extract 4-100.—Finely ground velvet bean seed previously dried at 40-45°C. was extracted in 400 gm. portions with seven 1 liter fractions of hot alcohol (93 per cent by weight). Each fraction was filtered while hot through a large Buchner funnel. The alcohol was distilled off under diminished pressure until the extract began to thicken. This was then washed out of the flasks with a little distilled water, and the several fractions were collected in a separatory funnel until a total of 2 kilos of seed was extracted.

Anhydrous ether (500 cc.) was then poured into the funnel to dissolve the extracted fat which had collected on top of the concentrated extract and the layers were separated. The aqueous layer was returned to the funnel and shaken with 1 liter of fresh ether and finally with another 500 cc. portion. This treatment resulted in a clear aqueous layer (varying in color from straw to reddish brown with different samples of beans) separated from the ethereal layer by a thin layer of white precipitate. The aqueous layer was drawn off and filtered free of the precipitate. Acetic acid to 0.10 per cent was added to the approximately 1 liter of extract which was heated on the water bath for a short time to drive off the remaining ether.

After the solution was cooled a saturated solution of lead acetate was added and the voluminous, flocculent precipitate filtered out. The excess lead was removed from the filtrate with dilute H_2SO_4 . The solution was filtered twice and 50 gm. of fullers' earth were added. The suspension was stirred intermittently for about 30 minutes, allowed to stand overnight, stirred again, and the fullers' earth filtered out on a Buchner funnel. The solid was washed several times with distilled water and finally with 93 per cent alcohol. It was then dried over H_2SO_4 in a partially evacuated desiccator. Two lots of 2 kilos each were treated this way and the two portions of the "activated solid" combined.

Extract 4-25 — This was prepared by the same method as Extract 4-100 except that only 25 gm. of fullers' earth were added to the extract from 4 kilos of velvet beans.

Extract 2-10.— This was prepared as above with 10 gm. of fullers' earth used for each 2 kilos of velvet beans.

Extract L-2-100.—Velvet bean leaves were gathered from the field and allowed to dry on the floor of an attic room. 2 kilos of the air-dry material were extracted twice on a boiling water bath with 10 liters of tap water acidulated to 0.10 per cent with acetic acid. Each extraction was continued for about 1 hour and the decoction was pressed out in a wine press. The extract was strained through heavy muslin, allowed to cool, and 100 gm. of fullers' earth were then added. The mixture was churned for 30 minutes in a small power churn and allowed to stand overnight. The solid was filtered out on a Buchner funnel, washed with distilled H_2O and then with alcohol, and dried as above.

Extract L-1-50.—The method for this extract was similar to that used for Extract L-2-100. The velvet bean leaves were extracted in 500 gm. portions: first, with 5 liters of the acidulated water for each portion, and then with 4 liters for each portion. The decoction from 1 kilo was treated with 50 gm. of fullers' earth.

Extract L-1-500.—After the fullers' earth was filtered out in the preparation of Extract L-1-50, the filtrate from the 1 kilo of velvet bean leaves was evaporated under diminished pressure until the volume was reduced to about 3 liters. The concentrated solution was then treated with 20 gm. of fullers' earth. The solid was filtered out as before and the evaporation of the filtrate continued until the extract was reduced to a thick, brown syrup. This was poured into a flat pan, 500 gm. of starch were added, and the mixture was dried in an air oven at 45–50°C.

Measurement of Antineuritic and Growth-Promoting Values.

The original plan of the experiment purposed: (1) to measure the relative antineuritic values of the various substances by determining the minimum daily dose necessary to protect mature pigeons against loss of weight or development of symptoms of beriberi; (2) to determine the relative growth-promoting properties (or water-soluble B content) of the same substances by establishing the minimum daily allowance necessary to maintain the body weight and also the amounts required to permit normal growth of rats receiving an otherwise vitamin B-free diet

*Procedure for Pigeons.*¹—The essentials of the method used have been described elsewhere (5). The pigeons were fed by hand the following basal diet: one feed per day of polished rice² and one feed per day of mash mixture³ consisting of casein 20 parts, polished rice 71, agar 2, CaHPO_4 0.5, CaCO_3 1, NaCl 0.5, butter fat 5.

The substance to be tested for its protective action was forced into the crop of the pigeon by hand just before the feeding of the basal food. The daily allowance of the material being tested was

¹ Mr. G. J. Cottier helped the author in caring for the animals used in these tests.

² The polished rice was heated in an air oven for 6 hours at 120°C.

³ All the components of the mash mixture, except the butter fat, were autoclaved for 3 hours at 15 to 17 pounds pressure. The product was then dried, ground, and the butter fat added.

varied until the amount which would just prevent the disgorging of food and consequent loss of weight was established.⁴

The total daily allowance of food was calculated by the formula: $(300)^{\frac{1}{2}} : (\text{body weight in gm.})^{\frac{1}{2}} = 15 : (\text{gm. of food per day})$.

Procedure for Rats.—The usual laboratory routine for determinations of vitamin B was used. Rats 25 to 28 days of age and averaging 45 to 50 gm. in weight were placed in individual cages of a type which prevented access to the excreta. Fresh portions of the following basal diet (Diet 2B) were supplied daily: extracted⁵ casein 18 parts, salt (No. 186) 3.7, agar 2, corn-starch 69.3, cod liver oil 2, butter fat 5.

After the rats had received the basal diet for 2 weeks, the weight became practically stationary, due to the depletion of the vitamin stored in the body. The feeding of the material to be tested was then begun, weighed amounts being supplied separate from the basal diet each day, including Sunday. Careful records of the total food intake were kept.

In all cases where extracts were being tested the jars containing the basal diet were placed in the cages late in the afternoon, left overnight, and removed early the next morning, at which time the extracts were supplied. This was found to result in a more uniform eating of the extracts.

Results.

Relative Values of Plant Materials.

Relative Antineuritic Values for Pigeons.—Pigeons weighing 300 to 370 gm. were afforded complete protection by 2.4 to 2.7 gm. of velvet beans or soy beans (seed) per day (Table I). In all cases, however, the feeding of 3 gm. or less of the dried leaves of the

⁴ If the dosage given was too low and the pigeon developed polyneuritis enough dried yeast (usually about 4 gm.) was given to alleviate the symptoms. The bird was then continued on the experiment.

⁵ The casein was extracted with 0.20 per cent acetic acid, then with distilled H₂O, and finally with 93 per cent (weight) alcohol to facilitate drying. Salt 186 was prepared by adding 0.004 per cent of KI to McCollum's Salt Mixture 185. The butter was churned from freshly soured cream obtained from the station dairy. It was washed, melted, filtered clear as usual, placed in small jars, and kept in a refrigerator until used. Squibb's cod liver oil was used.

velvet bean or of rape led to a rapid development of severe polyneuritis or experimental beriberi. In fact, 2 gm. of soy beans per day prevented the onset of the disease for considerably longer periods than 3 gm. of the leaves. The exact minimum protective dose of the leafy materials was not established but it was apparently near 3.4 to 4.0 gm. of the dried material per day.

Relative Maintenance Values for Rats.—The comparative values of small amounts of the seeds and of the leaves for prolonging the life of rats, receiving the basal diet, also indicate significant differ-

TABLE I
Relative Antineuritic Values of Seeds and Leaves.

Material tested	No of birds used	Average length of period	Daily dose of dry substance	Average gain + or loss —	Condition of bird.
		<i>days</i>	<i>gm</i>	<i>gm</i>	
Velvet bean seed	2	49	2 4	—2	Normal.
	1	56	2 6	+44	"
	1	112	2 7	+21	"
	2	17	3 0	+37	"
Soy bean seed.	2	42	2 0	—30	Beriberi
	2	70	2 4	—10	Normal
	2	49	2 6	+28	"
Velvet bean leaves.	1	21	2 6	—31	Beriberi
	3	14	3 0	—35	"
	1	28	3 4	—4	Normal
	1	84	4 0	+11	"
Rape leaves.	1	11	2 4	—5	Beriberi.
	2	21	2 7	—53	"
	1	14	2 7	—13	Disgorging.
	1	21	4 0	+16	Normal

ences in the properties of the two classes of materials (Table II). The rats that received 0.10 or 0.15 gm. of soy beans lived nearly twice as long as those that received corresponding amounts of rape leaves. On a dosage of 0.20 gm. per day the difference was not as marked as on the smaller amounts but was still great enough to be significant. The combination of soy beans 0.10 gm. and rape 0.10 gm. or of velvet beans 0.10 gm. and rape 0.10 gm. was more efficient than a corresponding amount of rape alone, but not as

efficient as 0.20 gm. of soy beans. The rats that received the velvet bean leaves were not litter mates of the rats in the other groups (although of the same stock and reared on the same diet) and, therefore, not strictly comparable; yet it is striking that the rats on 0.25 gm. of velvet bean leaves per day lived less than half as long

TABLE II
Relative Maintenance Values of Plant Materials.

Litter.	No. of rats.	Daily dose of dry material.	Average weight of rat at end of preliminary period	Average gain + or loss -.	Average length of life *	Average total daily food †
		<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>days</i>	<i>gm</i>
591-2	2	0 10 soy beans.	63 2	-27 2	90	2 45
	2	0 15 " "	59 2	-19 5	112‡	3 02
	2	0 10 rape.	65 2	-21 0	53 5	2 26
	2	0 15 " "	64 7	-16 7	54 5	2 66
402-5	2	0 20 soy beans.	56 0	+3 7	143 0§	3 31
	2	0 20 rape.	56 5	+1 2	108 0	3 16
	2	{ 0 10 soy beans. 0 10 rape.	60 2	-1 7	128 5§	3 28
	2	{ 0 10 velvet beans. 0 10 rape.	60 2	+2 0	133 0§	3 47
	4	0 15 velvet bean leaves.	45 6	-9 4	29 3	3 23
320-1	4	0 15 velvet bean leaves.	45 6	-9 4	29 3	3 23
350-6, 351-5	4	0 25 velvet bean leaves	52 0	-3 1	41 0	4 54
	5	Negative controls.	50 2	-15 3	16 0	2 10

* Does not include the 2 week preliminary period on the basal diet alone.

† Includes vitamin-carrying material being tested.

‡ One rat destroyed at end of 126 days.

§ One rat destroyed at end of 154 days.

as those on 0.10 gm. of soy beans and less than one-third as long as those on 0.20 gm. of soy beans per day.

It seems significant that in practically every case where our rats have been fed 0.25 gm. or less of the leafy materials per day as the sole source of the so called vitamin B they have developed severe cases of polyneuritis or beriberi. This has been characterized by stiffness, a reeling gait, incoordination of muscles, and

finally by complete prostration and death. The negative controls, on the other hand, have rarely developed such symptoms. Neither have these symptoms been prevalent among the rats that received the seeds, except in the case of the animals that received as little as 0.10 to 0.15 gm. daily for long periods of time. We have noticed, however, a tendency of the animals that received small allowances of the seeds to lose their fur in patches, and in a few cases an inflammation of the eye, which was suggestive of xerophthalmia, has been noted. One rat, receiving 0.20 gm. of soy beans per day, developed an ophthalmia so severe that by the 18th week of the

TABLE III
Relative Growth-Promoting Values of Seeds and Leaves.

Litter.	No of rats	Daily dose of dry material	Length of test *	Average weekly gain per rat	Average total daily food per rat †	Average total food for 1 gm gain
		gm.	wks.	gm	gm.	gm
348-7	2	1 0 soy beans.	6	14 1	6 52	3 23
	2	2 0 " "	6	15 0	7 06	3.29
	2	1 0 rape.	6	19 4	8 66	3 15
	2	2 0 " "	6	25 7	11 07	3.00
350-6	4	1 65 velvet bean leaves.	4	22 2	11 61	3 65
351-5	4	0 63 yeast	6	19 1	10.72	3 92
300	4	1.85 velvet beans (seed).	6	10 0	9 27	6 49

* Does not include the 2 week preliminary period on the basal diet alone

† Includes vitamin-carrying material being tested.

test proper one eye was completely closed. At that time this animal was consuming in the basal diet an average of 150 mg. of butter fat and 60 mg. of cod liver oil per day. The shedding of the fur or the eye trouble has not been noted among the rats that received the leaves.

Relative Growth-Promoting Values for Rats.—Portions of the same samples of materials that had been used in the previous tests were tested also for their growth-promoting action (Table III). The rats which received 1 or 2 gm. of rape leaves per day decidedly outstripped their litter mates which received 1 or 2 gm. of soy beans per day.

Young rats will not consume as much as 1 or 2 gm. of velvet bean seed or leaves per day, if these are fed apart from the basal diet. These materials were, therefore, incorporated into the basal diet, 20 per cent of the seed and 15 per cent of the leaves respectively replacing an equal amount of starch in the diets. Again the superiority of the leafy material as a growth-promoter is demonstrated, a daily intake of 1.65 gm. of the leaves producing more than twice the growth produced by 1.85 gm. of the seed. The growth-promoting action of 1.65 gm. of velvet bean leaves or 2 gm. of rape per day seemed to be even superior to that of 0.40 or 0.50 gm. of dried brewers' yeast per day.

Relation of Above Values.—There is apparently no correlation between the antineuritic and the growth-promoting values of the two classes of materials tested in the experiments described in the preceding pages. The seeds are more potent than the leaves in their antineuritic value for pigeons and their protective or maintenance value for rats. In contrast with this, the leaves show a greater growth-promoting action than the seeds. There does seem to be, however, a direct relation between the potency of the materials in protecting pigeons against polyneuritis and their efficacy in maintaining the weight of rats and protecting them against polyneuritis or a similar syndrome.

These results seemed to indicate that vitamin B is a complex consisting of at least two factors, the proportions of which may vary in the seeds and leaves. We, therefore, undertook the second phase of the investigation with the idea of trying to separate these factors.

Relative Values of Various Extracts.

The details of the preparation of the extracts have been described under "Methods." In the previous experiments it had seemed that the velvet bean (seed) was relatively rich in the antineuritic factor and poor in the growth factor. It seemed possible, therefore, that a fraction might be obtained from this source which would have antineuritic action and at most only slight growth-promoting action. Three preparations, Extracts 4-100, 4-25, and 2-10, of the fraction which is adsorbed by fullers' earth have been obtained from this source. Two fullers' earth fractions, Extracts L-2-100 and L-1-50, have been prepared from extracts of

TABLE IV.

Amount of Plant Material Extracted, Substance Added, and Dry Weight of Extract Recovered

No of extract	Material extracted	Fullers' earth added	Starch added	Average total dry weight of extract	Original material represented by 1 gm of extract *
	gm	gm	gm	gm.	gm
	Velvet beans (seed).				
4-100	4000	100		96 10	40 0
4-25	4000	25		23 75	160 0
2-10	2000	10		9 60	200 0
	Velvet bean leaves				
L-2-100	2000	100		95 0	20 0
L-1-50	1000	50		58 0	17 24
L-1-500			500	732 0	1 36

* On basis of fullers' earth added, except for Extract L-1-50, it was assumed that the solid which was lost would carry out its proportion of vitamin.

TABLE V

Antineuritic Values of the Extracts for Pigeons

No of extract			No of birds used	Average length of period	Daily dose of extract	Average gain + or loss -	Condition of bird
				days	gm	gm	
4-100,	fullers' earth		2	8	0 30	0	Disgorging.
"	"	"	3	14	0 40	+12	Normal.
4-25	"	"	1	28	0 12	+8	"
"	"	"	3	13	0 15	+18	"
2-10	"	"	2	31	0 10	+22	"
L-2-100	"	"	2	14	0 36	+5	"
"	"	"	2	7	0 20	-22	Disgorging
L-1-50	"	"	2	7	0 36	+14	Normal.
"	"	"	2	7	0 34	+3	"
"	"	"	2	14	0 22	-20	Disgorging.
L-1-500 residue			2	7	6 0	-12	"
"	"		1	14	7 00	-22	"
"	"		1	14	8 00	-40	Beriberi.

velvet bean leaves. Also one residue, Extract L-1-500, has been prepared by evaporating the filtrate, after two successive treatments of an extract of velvet bean leaves with fullers' earth and removal of the solid. The amount of material represented by 1 gm. of the various extracts is shown in Table IV.

Antineuritic Values for Pigeons.—The protective values of the various extracts for pigeons are indicated in Table V. Complete protection was afforded by the following daily doses of the acti-

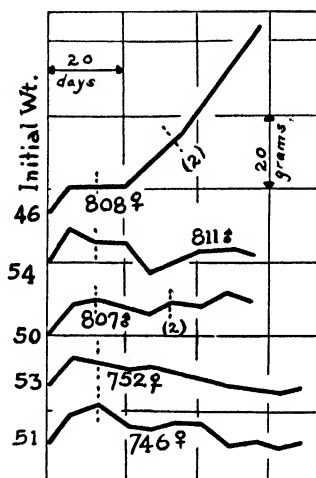


FIG. 1. Representative weight curves of rats. All rats received only the basal diet during the first 2 weeks. Rat 746 received 0.05 gm. and Rat 752 0.50 gm. per day of Extract 4-100. Rat 807 received 0.01 gm. per day to point (2) and then 0.012 gm. per day of Extract 2-10. Rat 811 received 0.05 gm. of Extract 2-10 per day. Rat 808 received 0.01 gm. of Extract 2-10 plus 0.25 gm. of Extract L-1-500 per day to point (2) and then 0.012 gm. of Extract 2-10 plus 0.25 gm. of Extract L-1-500 per day.

vated fullers' earth: 0.40 gm. of Extract 4-100, 0.12 to 0.15 gm. of Extract 4-25, 0.10 gm. of Extract 2-10, or 0.34 to 0.36 gm. of Extracts L-2-100 and L-1-50. On the other hand the residue, Extract L-1-500, failed to offer any demonstrable protection even in daily doses of 7 to 8 gm.

Values of Extracts for Rats.—The effects of feeding the various extracts to rats are shown by the typical weight curves in Figs. 1 and 2. Young rats approximately maintained their weight for 6

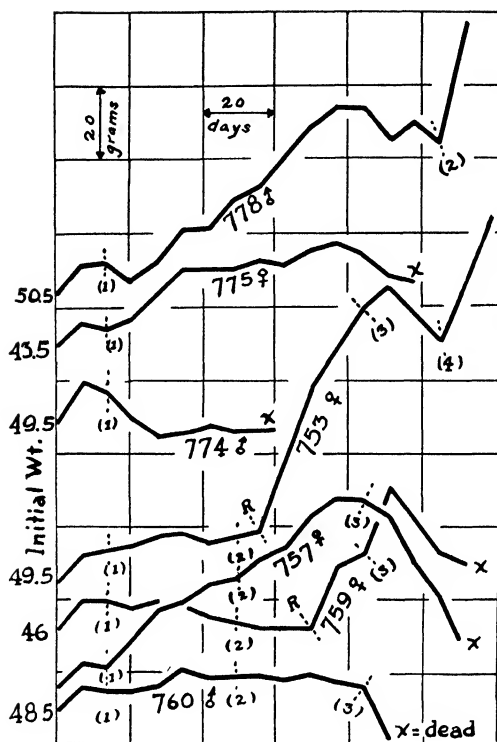


FIG. 2. Representative weight curves of rats. All rats received only the basal diet during the first 2 weeks. Rat 760 received 0.05 gm., Rat 759 0.05 gm., Rat 757 0.40 gm., and Rat 753 0.10 gm. of Extract L-2-100 per day during the period (1) to (2). These four rats received the same daily amounts of Extract L-1-50 during the period (2) to (3). At point (3) Extract L-1-50 was discontinued leaving Rats 760 and 757 on the basal diet only and Rats 759 and 753 on the basal diet plus 0.50 gm. daily of the residue, Extract L-1-500, which they received from point R to close of test. Rat 753 was also given 0.05 gm. of Extract 2-10 per day from point (4). Rat 774 received 0.25 gm., Rat 775 0.50 gm., and Rat 778 1.00 gm. per day of the residue, Extract L-1-500. Rat 778 received 0.05 gm. of Extract 4-25 per day from point (2) in addition to the residue.

to 8 weeks on daily allowances of 0.05 gm. of Extract 4-100, 0.012 gm. of Extract 2-10, or 0.05 gm. of Extracts L-2-100 and L-1-50. A daily allowance of 0.25 gm. of the residue, Extract L-1-500,

was not sufficient for maintenance. When the residue was increased to 0.50 or 1.00 gm. per day there was maintenance of weight or slight gains for about 8 weeks. The rats then began to decline, some of them developing symptoms of beriberi. The further addition of the activated fullers' earth at this point resulted in marked gains in weight and the disappearance of the symptoms of beriberi.

Whether the rats could have maintained their weight for much longer periods on the fullers' earth fraction from the velvet bean seed as the sole supplement to our Diet 2B is not shown by these experiments. There was in most cases a slight downward trend to the weight curves but, after 8 to 10 weeks on that diet with no supplement except the fullers' earth fraction, the rats were in fair condition. The only abnormalities noticed were a somewhat listless behavior, rather dry coats, a marked tendency to scatter the basal diet, and in a few cases the appearance of a slight dried secretion on the lids of one or both eyes, which caused the lids to adhere together. The latter condition seemed to suggest the incipient symptoms of a vitamin A deficiency which we could not explain. The recent paper by Goldberger and Lillie (3), however, seems to throw some light not only on this condition but also on the ophthalmia and the loss of fur by our rats that were maintained for long periods on small dosages of the seeds.

No one fraction of the various extracts, when used as the sole supplement to the basal diet, produced marked growth. Daily allowances of Extract 4-100 which were 2, 4, or 10 times the maintenance dosage did not cause any growth. Correspondingly large dosages of the fullers' earth fraction from the other extracts of the velvet bean seed also failed to produce growth. Relatively large dosages of the fullers' earth fraction from extracts of velvet bean leaves or of the residue obtained by evaporating these extracts after treating them with fullers' earth caused an indifferent and irregular growth. It was only by a combination of the activated fullers' earth and the residue that a rate of growth approaching normal was obtained. Normal growth was produced by 0.50 gm. of the residue per day in addition to a daily dosage of the activated fullers' earth which was slightly in excess of the minimum maintenance requirement.

DISCUSSION.

The data presented in the preceding pages show a marked lack of correlation between the antineuritic and the growth-promoting properties either of the plant materials studied or of fractionated extracts from some of these materials. Apparently the plant products contained varying proportions of at least two active substances. A still greater variation in the proportions of these substances was found in the different fractions of the extracts. When the extracts were treated under certain conditions with small amounts of fullers' earth, the resulting activated solid protected pigeons or rats against experimental beriberi but did not suffice for the growth of rats on an otherwise vitamin B-free diet. On the other hand after an acidulated aqueous extract of velvet bean leaves had been successively treated with two small portions of fullers' earth and the filtrate evaporated to dryness, the residue possessed, at the most, extremely weak antineuritic or beriberi-preventing properties. Furthermore the residue alone was not very efficacious in promoting growth of the rat. However, when the residue was added to small amounts of the activated fullers' earth it was very potent in its growth-promoting effect.

It seems then that vitamin B, as it has most often been demonstrated in the various natural foods, is a complex consisting of at least two substances or groups: (1) a specific factor which prevents the occurrence of experimental beriberi in pigeons and rats, and (2) another factor which does not prevent the occurrence of beriberi and is not in itself capable of inducing growth but which is a very potent promoter of growth when it is added to the beriberi-preventing factor.

Since the term water-soluble B, or vitamin B, was originally proposed for what seems to be a combination or a mixture of two or more substances and since the term vitamin D, proposed by Funk, is also used to designate the antirachitic factor, it may be desirable to retain the term *vitamin B as referring to the complex*. In this case it is suggested that the specific factor which prevents polyneuritis or experimental beriberi be tentatively designated as *vitamin B-P* or the beriberi-preventing factor.

Any speculation as to whether the growth-promoting substance is identical with the factor P-P or pellagra-preventing factor would be premature at this time. The elucidation of this question

must await the results of further studies. It would seem, therefore, that a separate designation should not be proposed for the growth-promoting factor unless the results of such studies prove it to be non-identical with the factor P-P.

SUMMARY.

1. Comparative tests on seed of the velvet bean and the soy bean, and on leaves of the velvet bean and of rape have shown a higher antineuritic or beriberi-preventing value for the seeds than for the leaves.

2. The tests have shown the leaves to be more potent than the seeds in their growth-promoting action.

3. A fullers' earth fraction (activated solid) which prevented experimental beriberi or polyneuritis of pigeons and rats but which did not induce growth of rats has been prepared.

4. Another fraction (the residue) which possessed extremely weak antineuritic or growth-promoting action when fed alone but marked growth-promoting action when added to the antineuritic fraction, has been obtained.

5. The results have been interpreted as indicating further that the so called vitamin B is a complex containing two or more active substances

6. It has been suggested that the term *vitamin B* be retained to designate the complex.

7. It has also been suggested that the specific factor which prevents polyneuritis or experimental beriberi be designated as *vitamin B-P*, or the beriberi-preventing factor.

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AN APPARATUS AND METHOD FOR THE DETERMINATION OF THE RESPIRATORY QUOTIENT OF SMALL ANIMALS.

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The usual gas analytical methods for determinations of the respiratory quotients of small animals were found to be inadequate for the purpose of the work described in the accompanying paper.¹

One reason for this was the fact that the quotients to be measured were assimilative and widely fluctuatory instead of basal and approximately constant. Also it was especially desirable to be certain that the highest value in the assimilative period was included, and, if only low values were obtained, that high values had not been missed. In fact, the conclusions drawn from these quotients were based upon the fact that high values, that is values appreciably above 1, had or had not been obtained.

For these reasons, the apparatus and method described in the following paragraphs were developed and used in place of the available gas analytical methods.

The apparatus is of the closed circuit type, the air being circulated by means of a single-acting, oil-sealed,² piston pump (*E*, Fig. 1) run by a motor at a speed of 12 cycles per minute. Each down stroke of the pump propels about 80 cc. of air through the 1 liter chamber (*D*) which contains the animal, and into a 6 liter flask (*B*, air storage flask). Each up stroke of the pump sucks about 80 cc. of air from this flask directly into the barrel of the pump without its passing through the animal chamber. The direction

¹ Wesson, L. G., *J. Biol. Chem.*, 1927, lxxiii, 507.

² A heavy grade of machine oil was prepared for this purpose by heating it to 100°C. *in vacuo* for 1 hour

which the air takes is determined by a rotary valve (*F*) attached to the shaft of the crank wheel (*G*) of the pump.

The CO_2 breathed out by the animal is removed from the air which comes from the animal chamber or jar by means of a spiral scrubber (*H*) moistened with standardized NaOH solution (approximately normal). This solution is contained in a circular well

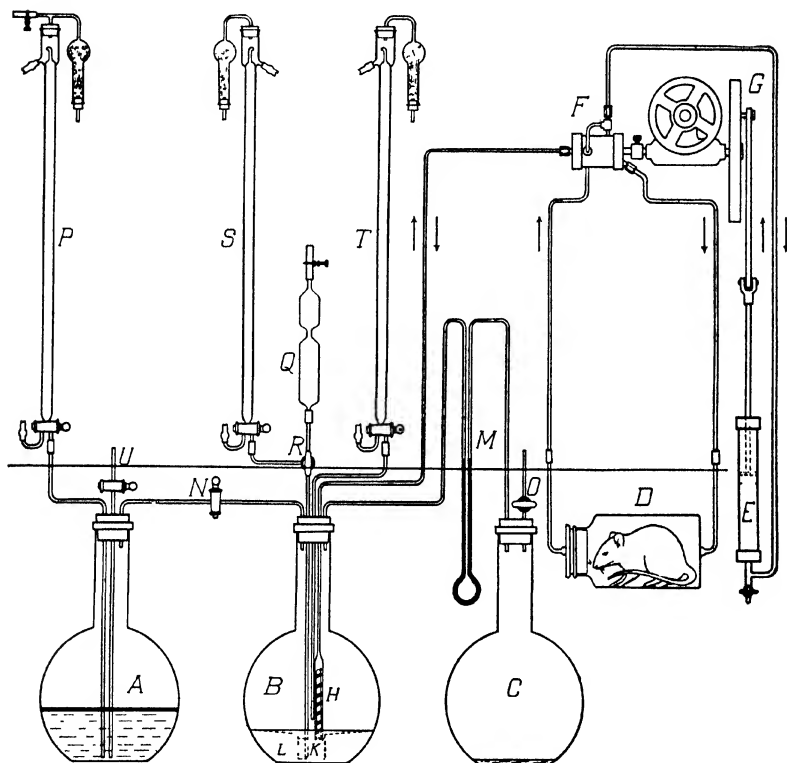


FIG. 1.

(*K*), 5 cm. in diameter by 5 cm. deep, cut in a solid block of paraffin (*L*) which fills the bottom of the air storage flask. The spiral scrubber (*H*) through which air both enters and leaves the flask is kept moistened with fresh NaOH solution in the following way. When air is drawn from the flask by each up stroke of the pump, liquid is at first sucked up with it to the top of the scrubber (*H*)

because the end of the scrubber dips a few mm. below the surface of the solution. When on the down stroke of the pump air is forced into the flask, it passes through the same tube and scrubber by which it left the flask, and thus over a large, freshly moistened surface. In this manner satisfactory absorption of CO_2 is obtained. The amount of CO_2 absorbed in a given period of time is determined at the end of that period by withdrawing exactly 50 cc. out of approximately 55 cc. of the alkali and titrating with standardized HCl in the presence of an excess of BaCl_2 solution and phenolphthalein.

As O_2 is used by the animal, the gas pressure in the system diminishes, as is indicated by a water manometer (M) on the air storage flask. A compensatory flask (C) is connected to the other arm of this manometer. This compensatory flask is provided with a stop-cock (O) which opens to the air of the room and which is closed at the beginning of the run. The compensatory flask is thus entirely shut off throughout the duration of a run. The *initial* temperature and the *initial* barometric pressure at this time are recorded and are used in the calculations. Moreover, as the compensatory flask contains a small amount of the NaOH solution, such as is used in the air storage flask, the change of water vapor pressure in it will compensate for any changes of the water vapor pressure in the system due to changes of temperature during the run. In other words, this is the same mechanism for compensation that is used in the Haldane gas analysis apparatus.

When enough O_2 has been used to affect markedly the swing of the manometer with the alternate motions of the pump, more O_2 is admitted to the air storage flask during the suction stroke from a third flask of equal size which we will call the oxygen storage flask (A). As O_2 is required, water is admitted from a burette (P) to displace it from the oxygen storage flask. At the beginning of a period the pressure of the oxygen in the oxygen storage flask is adjusted to be equal to the pressure of the air in the air storage and the compensatory flasks, by admitting water to the oxygen storage flask in the right amount to bring the gas pressure in the oxygen storage flask and the air storage flask just equal to the pressure in the compensatory flask. Similar adjustment is made at the end of each period. The volume of O_2 used is measured by the volume of the water which must be admitted to the oxygen

storage flask to take the place of the O_2 which is absorbed from the beginning to the end of each and every period.

The water in the oxygen storage flask is covered by a layer of paraffin oil (also the water in the water reservoir is so covered) to minimize gas exchange between the water and oxygen (or air), the same water being used repeatedly. The oxygen storage flask is filled with oxygen through the burette (*P*), the water escaping by way of outlet tube (*U*) to the water reservoir, which for this purpose is temporarily placed at the level of *A*.

In order that the temperature may be practically the same throughout the system, thereby minimizing errors from that source, the three flasks and the animal chamber are completely immersed in a well stirred water bath.³ A temperature reading of the bath is made at the beginning of the run, and, by the use of it and the initial barometer reading, the oxygen volume is computed for standard conditions.⁴

A run, that is to say, a series of successive determinations of the respiratory quotients, is made as follows. A weighed animal is placed in the animal chamber, and this is connected to its place under the surface of the water. 50 cc. of NaOH solution are run from burette (*T*) into the paraffin well of the air storage flask which already contains approximately 5 cc. of the residual solution from the last run. The outlet of the compensatory flask is closed and the barometer is read. The pump is now operated for a preliminary period (say 1 hour) to allow all parts of the system and the animal to come to approximate equilibrium. At the end of this preliminary period, the pump is stopped at a point in its stroke that fixes the volume of air in the cylinder of the pump and also leaves the animal chamber open to the system. The stop-cock (*N*) connecting the oxygen storage flask with the air storage flask is opened, and water is admitted from the burette until enough O_2 has been displaced from the oxygen storage flask to make the gas pressures in these two flasks equal the pressure in the compensatory flask. 50 cc. of the NaOH solution are then withdrawn by suction into a pipette (*Q*), the capillary tube above the 3-way

³ An ash can 20 inches in diameter and 26 inches deep was used for this purpose

⁴ Boothby, W M, and Sandiford, I, Laboratory manual of the technic of basal metabolic rate determinations, Philadelphia, 1920, 96-105.

stop-cock (*R*) rinsed with water (from *S*) into this pipette, forcing part of the alkali into the upper bulb of the pipette. The alkali and washings are transferred to an Erlenmeyer flask and the pipette rinsed with water. 50 cc. of a 10 per cent BaCl_2 solution are then added to precipitate the carbonates, and the remaining alkali is titrated with standardized HCl in the presence of phenolphthalein (Winkler's method). In the meantime 50 cc. of fresh alkali (from burette *T*) are run into the paraffin well, the pump started, and thus a new period is begun.

Calculations.

Example.—Rat 4, after 24 hours complete fasting following a normal diet, was placed in the calorimeter April 30. The data obtained were as follows:

Weight 250 gm ; barometer 750 mm ; temperature 21.5°C

CO_2 value of the approximately normal HCl solution, 1 cc = 11.38 cc.
 CO_2 at 0° and 760 mm

Titration value of the NaOH solution, 50 cc. = 33.85 cc. HCl solution.

Period	$\text{O}_{21.5^\circ}^{750 \text{ mm}}$	Titration
	cc	cc
Preliminary		18.7
11.35 a m – 12.05 p m	272	16.8
12.05 p m – 12.35 “	268	17.4
12.35 “ – 1.05 “	260	17.6

First Period.—Calculation of the carbon dioxide: The approximately 5 cc. of NaOH solution left in the flask at the end of the preliminary period were equivalent to one-tenth of the titration value for the 50 cc. of NaOH solution which were withdrawn, or were equivalent to 1.87 cc. of HCl . As 50 cc. of fresh NaOH solution had been added at the beginning of the first period, the total alkali in the flask at the *beginning* of this period was equivalent to $1.87 + 33.85$ or 35.72 cc. of HCl . At the *end* of the first period, 50 cc. of the used NaOH solution require 16.8 cc. of HCl for neutralization; therefore the total NaOH in the flask at this time would require $16.8 + 1.68$ or 18.48 cc. of HCl . The difference between the titration values at the *beginning* and at the *end* of the first period, or 17.24 cc. of HCl , is equivalent to 17.24×11.38 or 196 cc. of dry CO_2 at 0° and 760 mm.

Calculation of the oxygen used: 272 cc. of O_2 at 21.5° and 750 mm. are calculated by means of tables⁴ to be 242 cc. of dry O_2 at 0° and 760 mm.

Calculation of the respiratory quotient: The ratio of the carbon dioxide exhaled to the oxygen used in this period is $\frac{1}{4}\frac{9}{11}$ or 0.81.

Second Period.—The 55 cc. of NaOH solution in the flask at the *beginning* of the second period require $33.85 + 1.68$ or 35.53 cc. of HCl solution, and at the *end* of the second period $17.4 + 1.74$ or 19.14 cc. of HCl solution. The NaOH therefore used is $35.53 - 19.14$ or 16.39 cc. of HCl solution. The CO_2 equivalent of 16.39 cc. of HCl is 16.39×11.38 or 187 cc. of CO_2 at 0° and 760 mm. As 268 cc. of O_2 at 21.5° and 750 mm. or 238 cc. of O_2 at 0° and 760 mm. were used, the respiratory quotient is calculated to be 0.78.

In like manner the respiratory quotient for the third period is calculated to be 0.80.

Metabolic Rate.—Having calculated the respiratory quotient for the first period of 30 minutes to be 0.79, one finds from tables⁴ that the number of calories corresponding to 1 liter of O_2 at 0° and 760 mm. and 0.79 R.Q. is 4.79 calories. As the volume of O_2 at 0° and 760 mm. in the first period is 0.242 liter of O_2 at 0° and 760 mm., the heat production in this period is 0.116 calories. For 1 kilo of body weight of rat and 1 hour, the value is 9.26 calories. Similarly, for the second period, the calculated metabolic rate is 9.10 calories, and for the third period, 8.86 calories; average 9.07 calories.

Standardization of the Apparatus.

The usual alcohol lamp method of calorimeter testing was found unsuitable for standardization, as the oxygen consumption of the smallest lamp that would continue to burn with alcohol was too great for the capacity of the apparatus. Caprylic alcohol and absolute ether were also tried.

An electrically heated combustion tube packed with platinized asbestos and placed in series with the animal chamber was used to obtain the combustion of the substance taken for the standardization. A minute unlighted lamp filled with absolute ether was placed in the animal chamber. The current of circulating air carried the ether vapors over the heated platinized asbestos and

TABLE I.
Respiratory Quotients and Metabolic Rates during Fasting Following a Normal Diet.

Period.	O ₂ ^{760 mm} ₀	CO ₂ ^{760 mm} ₀	R Q	Metabolic rate per kilo per hr.
Rat 5, after 24 hr. fast; weight 323 gm				
hrs	cc	cc		calories
0-0 5	231	194	0 85	10 4
0 5-1 0	248	197	0 80	11 1
1 0-1 5	254	208	0 82	11 4
1 5-2 0	245	190	0 78	10 9
2 0-2 5	238	185	0 78	10 6
2 5-3 0	229	179	0 78	10 1
3 0-3 5	228	175	0 77	10 0
3 5-4 0	226	180	0 80	10 0
4 0-4 5	223	174	0 78	9 9
4 5-5 0	233	185	0 79	10 4
Average			0 80	10 5
Rat 31, after 48 hr fast; weight 90 gm.				
0-1 0	187	146	0 78	9 9
1 0-2 0	165	126	0 76	8 7
2 0-3 0	170	106	0 78	9 0
3 0-4 0	174	132	0 76	9 2
4 0-5 0	209	157	0 76	11 0
Average			0 77	9 6
Rats 9 and 10, after 72 hr fast; combined weight 307 gm				
0-0 5	232	172	0 74	7 2
0 5-1 0	215	154	0 71	6 6
1 0-1 6	221	174	0 79	5 9
1 6-2 4	293	220	0 75	6 0
2 4-2 9	206	157	0 76	6 4
2 9-3 4	215	163	0 76	6 7
3 4-4 0	221	177	0 80	5 2
Average			0 76	6 3
Rats 11 and 12, after 72 hr fast; combined weight 304 gm				
0-0 2	99	74	0 74	7 7
0 2-0 6	170	126	0 74	6 9
0 6-1 0	206	149	0 72	7 6
1 0-1 3	150	114	0 76	7 0
Average			0 74	7 3
Rat 4, after 96 hr fast; weight 221 gm.				
0 0 5	178	158	0 71	8 6

the products of combustion through a copper coil immersed in ice water back to the distributing valve. After the preliminary period, a number of successive values for the carbon dioxide-oxygen ratio were obtained as follows: 0.72, 0.64, 0.67, and 0.66; average 0.67; calculated 0.67.

Table I gives data and calculated results of the respiratory quotients and metabolic rates of a number of fasting rats.⁵ Although the average of these results upon fasting animals and those obtained by ether combustion are close to the theoretical values, there is considerable variation in the single determinations. These variations were, however, not of sufficient magnitude to interfere in the experiments for which the apparatus and method were devised.

The apparatus was frequently tested for leaks by operating it for several hours with no animal in the chamber. This was done both with increased pressure and diminished pressure in the system. At other times the apparatus was allowed to stand overnight with either an increased or diminished pressure in the system. The manometer was used as an indicator.

⁵ Other examples of the use of the apparatus and method are given in the accompanying paper

A FAT FORMATION UNDER ABNORMAL CONDITIONS FROM CARBOHYDRATE BY THE RAT, AND ITS RELATIONSHIP TO A POSSIBLE, NEW DIETARY FACTOR.

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(Received for publication, February 17, 1927)

The observations to be described in a preliminary way in this paper are those of the formation of fat from carbohydrate under conditions which differ widely from those in which this phenomenon is commonly observed.

It is well known that when an easily absorbed carbohydrate is fed to a normal, well nourished animal, the storage capacity of the animal for carbohydrate is soon reached, and the excess of carbohydrate is converted into fat to be stored and used as such.

In the present paper a conversion of ingested carbohydrate into fat is described which, it is thought, may be attributed to a dietary deficiency, in contrast to the cause of the above mentioned, commonly observed fat formation under normal conditions.

Briefly outlined, this abnormal conversion of carbohydrate into fat may be shown as follows. A rat is placed upon a diet of dextrin and water for a period of several weeks. It is then fed a definite amount of dextrin, and its respiratory exchange is examined during the period of assimilation and storage of this dextrin. While normally the quotients thus obtained on an undernourished animal remain at or below 1, the quotient for carbohydrate combustion, the quotients obtained under these severe conditions may rise definitely above 1 for a short or long interval, in which case they are therefore abnormal. As a respiratory quotient above 1 is thought to indicate a conversion of carbohydrate to fat, we probably are now observing the conversion of a portion of the ingested carbohydrate into fat in spite of the general malnutrition of the animal.

If the respiratory quotients in the above test do not rise above 1 the rat is returned to the dextrin-water diet for another week and then retested during the assimilation of a test meal similar to the above. When abnormal values are obtained, tests made at intervals of a few days will in general show increasingly high quotients, indicating thus an increasing tendency to convert carbohydrate into fat.

In the above procedure, it is necessary to remove the possibility that conversion of carbohydrate into fat has not occurred because of the overfilling of the glycogen stores. It is, therefore, desirable to have a fasting period for the rat precede each and every dextrin test meal, and this course has, in the main, been pursued in the following experiments.

It should be stated, however, that many of the rats that are placed on this restricted diet die before they exhibit the fat formation just described. Especially is this true of rats that have been tested in November and December in contrast with those tested during the summer months. Specifically, the highest respiratory quotient obtained in these 2 months was 1.5, and the average maximum values 1.2 to 1.5, whereas in the summer and early fall months values well above 2 were frequently obtained. Moreover, values definitely above 1 were obtained with only 50 per cent of the rats which were put on the restricted diet during November and December, as contrasted with fully 75 per cent of the rats in the earlier season. These lower respiratory quotients do not, however, invalidate the conclusion arrived at in this paper, as they are sufficiently above 1 to indicate definitely fat formation. Experiments were in progress to ascertain if possible the source of this variation, when a communication by Cori and Cori¹ appeared in which a seasonal variation in the fat and carbohydrate metabolism of rats is described. This has led us to believe that, in spite of the above mentioned difficulty in the exact duplication of our summer months' values, a preliminary announcement of our results at this time is warranted.

Since it was found that a considerable period of restricted diet was necessary before the abnormal behavior toward carbohydrate became manifest, it seemed logical to assume that the rats would

¹ Cori, G. T., and Cori, C. F., *Proc Soc Exp Biol and Med*, 1926, xxiv, 123

not attain this condition upon the basic diet. The various components of the ration were then one by one tested in combination with dextrin to discover, if possible, which could be included with the dextrin to form a diet on which the rat would not attain this abnormality. It was found that rolled oats, casein, calcium carbonate, sodium chloride, and iodine could be included in the restricted diet, without preventing the appearance of the abnormal condition, although the effect of rolled oats was much to delay this.

It was also found that if lard, the remaining component of the diet, was fed to rats in the abnormal condition, in amounts much too small to have an influence as a metabolite, they gave normal respiratory quotients on the carbohydrate test meal for a number of days thereafter. As lard is usually a conglomerate representing a number of the tissues of the hog, the ether extracts of a few of these tissues were made and tested similarly to lard, some with positive and others with negative results. In a similar preliminary way, two vegetables of different nature were tested with positive results on the abnormal behavior with regard to carbohydrates.

These results indicate that under severe restriction of diet the intermediary metabolism of rats is so affected that the normal storage or utilization of carbohydrate is partially superseded by a direct transformation to fat. The fact that lard and other hog fat prevent this, suggests the possibility that the animals had become depleted of a substance which is necessary for the direct utilization of glucose.

EXPERIMENTAL.

Method.

Only the preferred method of preparing a rat for the tests described above will be given.

An adult rat (100 to 300 gm.) is placed in a separate cage, and allowed to feed *ad libitum* on dextrin (white, Merck's) with which has been incorporated 2 per cent by weight of C.P. CaCO_3 (to improve the rat's condition) and 2 per cent NaCl . The drinking water is distilled water to which has been added enough tincture of iodine to color the water to a barely perceptible degree (2 drops per liter). At the end of 2 or 3 weeks, the rat is made to fast for a day or so, in order to deplete somewhat the glycogen stores.

The rat is then fed 3 gm. of dextrin per 100 gm. of body weight, and as much of this dextrin as is not eaten voluntarily within an hour is made into a thin paste with distilled water and fed forcibly to the rat with a medicine dropper. The rat is then placed in the chamber of the calorimeter described in the accompanying paper,² and the respiratory quotient determined for each half hour to 1 hour period of the succeeding 7 or 8 hours. If the values found are not sufficiently high (1.5 and upward) the rat can be further fasted for a day or 2 thereafter, and another run made which will show the metabolism that takes place during the assimilation of the test meal. Or, in order to improve its condition, a mixture of dextrin 40 parts, rolled oats 60 parts, CaCO_3 2 parts, NaCl 2 parts, may be fed to the rat, followed by a day or 2 of fasting and the test meal of dextrin.

When respiratory quotient values of 1.5 or over are obtained, the rat is given, say, 1 gm. of the fat or 25 gm. of the vegetable which is to be tested for its effect on the glucose utilization, and 2 days later is given the test meal of 3 gm. of dextrin per 100 gm. of body weight. If the fat or vegetable has an effect which for present purposes we will term an "appreciable" effect, the respiratory quotient values obtained now after the last test meal will at no time rise above, let us say, 1.05, because the utilization of glucose by the rat has now become in this respect more nearly normal.

Calculations.

The respiratory quotients and metabolic rates have been calculated by the method outlined in the accompanying paper.²

No correction has been made for protein metabolism for two reasons. (1) Protein metabolism during the period of assimilation and utilization of a carbohydrate meal by an otherwise fasting animal is thought to be comparatively small in amount. (2) The extent of the protein metabolism is difficult to ascertain under the conditions of the experiment, and to obtain values at other times would interfere greatly with the sequence of events.

The metabolic rates are on the basis of calories per hour per kilo of body weight of rat. The periods for which the respiratory

² Wesson, L. G., *J. Biol. Chem.*, 1927, lxxiii, 499

quotient was less than 1.01 have been arbitrarily selected to represent an average metabolic rate for the entire duration of the run.

The left hand columns of the tables, representing the interval which elapsed after the test meal, have been calculated from the time at which the rat commenced eating to the middle of the period for which the respiratory quotient was obtained.

Restricted Diet.³

It was attempted to work out a diet which would in time induce in rats the abnormality in carbohydrate metabolism which is the subject of this paper, and which would, moreover, prolong the life of the animals as much as possible. A restricted diet which includes dextrin, iodine, calcium carbonate, sodium chloride, and *small amounts* of rolled oats is thought, at the present time, to be most effective in these respects.

Test Meals.

Except for the instances which are noted in the accompanying tables, test meals of dextrin, 3 gm. per 100 gm. of body weight of rat, have been given. In the other cases, 2 per cent calcium lactate has been added to the dextrin of the test meals. It is believed from the results of these experiments that a fasting rat will not ordinarily give quotients greater than 1 on a test meal of 3 gm. of dextrin per 100 gm. of body weight. This conclusion is quite apart from calculations of the utilization and storage capacity of the animals with respect to carbohydrate.

The body weight rather than the metabolic rate was chosen as a basis of calculation of the amount of the test meals because the metabolic rate, as determined in the chamber of the calorimeter, varies so much with the activity of the rat and the temperature of the calorimeter water bath.

Blood Sugar and Glycogen Determinations.

A number of blood sugar and several glycogen determinations were made without definite result.

³ See foot-notes of the tables for the restricted diets used

Discussion of Tables.

Of the twenty-seven rats, whose abnormal respiratory quotients have been used in arriving at the conclusions of the present paper, those obtained with ten have been given in an illustrative way in Tables I to III. The results obtained with the remaining seventeen rats, representing about 400 respiratory quotients, have, of necessity, been omitted because of lack of space.⁴

Respiratory quotients obtained with the twenty-seven rats that are included in the present work indicate:

1. A reduced rate of metabolism during the period of restricted diet is not the cause of the abnormal behavior with regard to carbohydrate metabolism, since high respiratory quotients were obtained in runs in which the metabolic rates were not relatively low, as is illustrated by Table II (Rats 26, 27, 28), Table III (Rat 30). Furthermore, Rat 15 (not shown in the tables) gave on a water diet maximum respiratory quotients of 1.00, 1.02, and 0.95, in three runs on the 10th, 12th, and 14th days. Since this rat lost 42 per cent of its body weight during the fasting period, and its metabolic rate became but one-third of its original rate as the result of the prolonged fast, the normal quotients which were obtained lead to the same conclusion.

2. The abnormal condition of the rats is not due to a deficiency of protein. This is indicated by the fact that casein and rolled oats in moderate amount do not prevent or cure the abnormal condition (Table I, Rats 6, 33, Table II, Rats 26, 27, 28, 32, 44; Table III, Rat 30).

3. The formation of fat from carbohydrate after the period of restricted diet is not due to the demand for fat in the metabolism of the rat. This is indicated by: (a) The onset of the abnormal condition apparently bears no relation to the loss in weight during the period of restricted diet (Tables I to III). (b) Extremely

⁴ An additional 17 rats were included in the work reported in this paper. Of these, 3 were preliminary, 5 died before a test run was made upon them, 3 died during the first run made upon them, 2 were discarded because of an uncertainty in regard to their restricted diet, 1 showed no high value in four runs up to the 30th day, 1 showed no high value in three runs up to the 18th day, 1 died during the forced feeding preliminary to the first run, and 1 (on a water diet) showed no high value in one run on the 6th day

TABLE I.
Rats on Restricted Diet.

Rat 5. Began restricted diet Apr. 27, 1926; weight 325 gm.			
4th day, cane sugar, 5 gm.		13th day, wt. 228 gm.	
5th day, wt. 269 gm		Time after test meal.	R Q
Time after test meal	R Q	hrs	
hrs.		1 8	1.08
0 3	0 76	2 3	1 01
0 8	0 74	2 8	1.26
1 3	0 76	3 3	1 05
1.8	0 78	3 8	0 99
2 3	0 80	4 3	1 02
2 8	0 82	4 8	0 97
3 3	0 89	5 3	0 96
3 7	0 86	5 8	0 99
4 3	0 89	6 3	0 97
6 8	0 91	6 8	0 93
7 3	0 90	7 3	1 04
7 8	0 90	7 8	0 98
Metabolic rate 6.8 calories.		8 3	0 94
		8 8	0 98
		Metabolic rate 7.7 calories.	
10th day, wt. 245 gm		14th day, wt. 206 gm.	
Time after test meal	R Q	Time after test meal	R Q
hrs		hrs	
3 2	1 01	2 6	1 07
3 7	0 97	3 1	1 08
4 2	1 02	3 7	1 10
4 7	1 01	4 6	0 97
5 2	0 96	5 1	1 17
5 7	0 98	5 6	1 17
6 2	0 95	6 4	1 10
6 7	1 03	7 2	1 20
7 2	0 97	7 7	1 15
7 7	0 98	8 2	1 14
8 2	0 93	8 7	1 22
8 7	0 91	9 2	1 13
9 2	0 90	9 7	1 10
9 7	0 89	10 2	1 06
Metabolic rate 8.7 calories		Metabolic rate 4.4 calories.	
		Died during night.	

TABLE I—Continued.

Rat 6 Began restricted diet May 10; weight 219 gm.			
4th-8th days, dextrin and CaCO ₃ * 9th day, casein, 5 gm 10th-11th days, dextrin, casein, CaCO ₃ , 5 gm daily † 12th day, dextrin, 5 gm		15th day, wt 178 gm	
		Time after test meal ‡	R Q
14th day, wt 179 gm		hrs.	
		3 2	1 33
Time after test meal ‡		4 1	1 32
		5 1	1 22
hrs		6 1	0 97
		6 8	0 95
		Metabolic rate 4 6 calories	
		16th day, wt 171 gm	
		Time after test meal ‡	R Q
		hrs	
		3 6	1 38
		4 4	1 39
		5 4	1 17
		6 5	1 13
		7 5	0 93
		Metabolic rate 4 5 calories	
		Died 19th day	

Rat 21 Began restricted diet July 13; weight 163 gm			
1st-13th days, dextrin		19th day, wt 115 gm	
15th day, wt 129 gm		Time after test meal ‡	R Q
Time after test meal	R Q	hrs	
hrs		2 8	0 95
2 9	0 87	3 8	1 39
3 5	0 88	4 8	1 25
4 3	1 00	Metabolic rate 5 4 calories	
5 0	0 97	21st day, wt 112 gm	
5 8	1 18	Time after test meal ‡	R Q
6 6	1 38	hrs	
7 5	1 06	2 6	1 01
8 3	1 05	3 6	1 02
9 2	1 05	4 6	0 95
Metabolic rate 5 8 calories		5 8	1 03
17th day, wt 126 gm ₁		* 6 9	1 26
Time after test meal ‡	R Q	Metabolic rate 5 4 calories	
hrs		After 21st day rat used for other purposes	
3 0	1 07	Killed 27th day for blood sugar and glycogen determinations	
4 0	1 09		
5 0	1 35		

TABLE I—*Concluded.*

Rat 33. Began restricted diet Aug. 11; weight 204 gm.			
1st-30th, 33rd-36th, 39th-43rd, 46th-48th, 50th-54th days, dextrin, rolled oats, CaCO ₃ , NaCl §		55th day, wt 134 gm	
38th day, wt 161 gm		Time after test meal	R Q
Time after test meal †	R Q	hrs	
2 3	0 94	2 8	1 02
3 3	0 94	3 8	1 00
4 3	0 92	5 1	1.06
5 3	0 89	6 3	0 97
6 3	0 88	7 3	0 87
7 3	0 89	8 3	0 93
Metabolic rate 5 8 calories		Metabolic rate 6 3 calories	
45th day, wt 148 gm		58th day, wt 128 gm	
Time after test meal †	R Q	Time after test meal	R Q
hrs		hrs	
3 0	0 99	2 8	1 19
4 0	1 01	3 8	1 51
5 0	1 00	4 9	1 07
6 0	0 92	5 9	1 52
7 0	0 90	7 0	1 60
8 0	0 86	60th day, wt 125 gm	
Metabolic rate 6 6 calories		Time after test meal	R Q.
49th day, wt 146 gm		hrs	
Time after test meal	R Q	3 3	2 76
hrs		4 5	1 90
4 1	1 02	5 7	1 11
5 1	0 91	7 0	1 09
6 2	0 89	Died 62nd day.	
Metabolic rate 6 1 calories			

* Dextrin 95 parts, CaCO₃ 5 parts† Dextrin 80 parts, technical casein 20 parts, CaCO₃ 5 parts

‡ See under "Test Meals" in text

§ Dextrin 40 parts, rolled oats 60 parts, CaCO₃ 2 parts, NaCl 2 parts.

TABLE II.

Rats on Restricted Diet and Later Small Amounts of Lard and Other Ether-Soluble Substances of Hog Liver, Heart, Kidneys, and Pancreas.

Rat 26 Began restricted diet July 27, weight 169 gm.			
1st-21st days, dextrin		24th day, wt 124 gm	
22nd day, wt 134 gm		Time after test meal *	R Q
Time after test meal	R Q	hrs	
2 6	0 98	5 1	1 08
3 6	0 90	6 1	1 05
4 6	0 92	7 1	1 01
5 6	0 94	8 1	0 99
Metabolic rate 3 8 calories		9 1	1 00
		Metabolic rate 4 9 calories Dextrin, rolled oats, CaCO ₃ , NaCl, 3 gm, after this run †	
23rd day, wt 130 gm		26th day, wt 131 gm	
Time after test meal *	R Q	Time after test meal *	R Q
hrs		hrs	
2 0	0 97	3 5	1 05
3 0	0 98	4 6	1 04
4 0	0 97	5 5	1 03
5 0	1 01	6 4	1 02
6 0	1 00	Ether extract of hog kidney, 1 gm, after this run	
7 0	1 01		
8 0	1 01		
9 0	0 99		
10 0	1 03		
Metabolic rate 4 7 calories		29th day, wt 110 gm	
		Time after test meal *	R Q
		hrs	
		3 0	1 24
		4 0	1 92
		5 0	1 87
		6 0	2 22
		7 0	1 18
		8 1	0 97
		9 1	0 95
		Metabolic rate 4 1 calories Died during night	

TABLE II—Continued.

Rat 27. Began restricted diet Aug. 4; weight 149 gm			
1st-16th days, dextrin		31st day, wt 120 gm	
17th-18th, 21st-22nd days, dextrin, rolled oats, CaCO ₃ , NaCl †		Time after test meal *	R Q
20th day, wt 134 gm		hrs	
Time after test meal	R Q		
hrs			
2 3	1 05	2 4	0 89
3 3	1 00	3 4	0 97
4 3	1 02	4 4	0 87
5 3	1 03	6 0	0 97
6 3	1 01	7 1	0 97
7 3	1 04	8 1	0 96
		9 1	0 99
Metabolic rate 5 2 calories		Metabolic rate 4 3 calories.	
24th day, wt 140 gm		34th day, wt 120 gm	
Time after test meal *	R Q	Time after test meal *	R Q
hrs		hrs	
3 3	0 99	2 9	1 02
4 3	0 94	4 1	1 24
5 3	0 97	5 2	1 32
6 3	0 94	6 2	1 45
7 3	0 97	Metabolic rate 4 6 calories	
8 3	0 87	Ether extract of hog heart, 1 gm ,	
9 3	0 90	after this run	
Metabolic rate 4 9 calories		36th day, wt 114 gm	
27th day, wt 130 gm		Time after test meal *	R Q
Time after test meal *	R Q	hrs	
hrs			
2 8	1 18	2 8	0 90
3 8	1 36	4 0	1 01
4 8	1 81	5 1	0 98
Ether extract of hog lungs, 1 gm ,		6 2	0 96
after this run		7 2	1 00
29th day, wt 127 gm		Metabolic rate 4 7 calories	
Time after test meal *	R Q	38th day, wt 108 gm	
hrs.		Time after test meal *	R Q
3 0	0 94	hrs	
4 1	0 98		
5 2	1 04	3 2	1 87
6 3	1 53	4 5	2 39
7 6	1 74	5 8	3 22
Metabolic rate 4 6 calories.		Killed for blood sugar and glycogen determinations	
Lard, 1 gm , after this run			

TABLE II—*Continued*

Rat 28 Began restricted diet Aug 4; weight 235 gm			
1st-16th days, dextrin.		29th day, wt 130 gm	
17th-18th, 21st-33rd days, dextrin, rolled oats, CaCO ₃ , NaCl †		Time after test meal *	R Q
20th day, wt 154 gm		<i>hrs</i>	
Time after test meal *	R Q		
<i>hrs</i>			
4 8	1 04	5 0	0 99
5 8	0 99	6 0	0 99
6 8	1 00	7 0	1 05
Metabolic rate 5 6 calories		8 2	0 91
		9 3	0 95
		Metabolic rate 5 2 calories	
25th day, wt 144 gm		30th day, wt 129 gm	
Time after test meal *	R Q	Time after test meal *	R Q
<i>hrs</i>		<i>hrs</i>	
2 3	0 95	3 1	0 91
3 3	1 01	4 1	1 02
4 3	1 08	5 1	0 91
5 3	1 12	6 1	1 02
6 3	1 00	7 1	0 99
7 3	1 04	8 1	1 06
Metabolic rate 6 1 calories		Metabolic rate 5 2 calories	
Ether extract of hog pancreas, 1 gm, after this run		Died during night	
27th day, wt 147 gm			
Time after test meal *	R Q		
<i>hrs</i>			
2 8	1 01		
3 8	1 04		
5 3	1 56		
6 0	1 43		
7 0	1 56		
8 2	2 11		
9 4	1 57		
10 4	0 94		
Metabolic rate 4 1 calories			
Ether extract of hog liver, 0 5 gm, after this run			

TABLE II—*Continued*

Rat 44 Began restricted diet Sept 28; weight 146 gm			
1st-14th days, dextrin, CaCO ₃ , NaCl ‡		19th day, wt 93 gm.	
16th day, wt 112 gm		Time after test meal	R Q
Time after test meal *	R Q	hrs	
hrs		2 5	0 98
3 2	2 20	3 6	Lost
4 2	2 57	5 0	1 04
5 2	1 35	6 0	0 99
6 2	0 99	7 0	0 99
Metabolic rate 4 0 calories		Metabolic rate 6 5 calories	
Ether extract of hog liver, 0 4 gm ,		Rolled oats, 3 gm , after this run	
after this run		20th day, wt 95 gm	
17th day, wt 110 gm		Time after test meal	R Q
Time after test meal *	R Q	hrs	
hrs		2 5	0 89
2 5	1 09	3 8	0 91
3 6	1 02	5 3	0 97
4 8	1 07	6 8	0 91
6 5	1 57	8 3	0 95
8 3	1 59	Metabolic rate 3 1 calories	
Metabolic rate 7 1 calories		Died during night	

TABLE II—*Concluded.*

Rat 32. Began restricted diet Aug 11; weight 243 gm			
1st-10th days, dextrin		52nd day, wt 154 gm	
11th-27th, 30th-36th days, dextrin, rolled oats, CaCO ₃ , NaCl †		Time after test meal	R Q
29th day, wt 213 gm		hrs	
Time after test meal *	R Q	2 8	1 99
hrs		4 0	1 13
3 3	0 94	5 1	1 22
4 5	1 00	6 3	1 30
5 5	0 97	7 6	1 02
6 5	0 95	8 7	0 87
7 5	0 89	Metabolic rate 2 9 calories	
Metabolic rate 4 4 calories		Ether extract of hog liver, 0 4 gm , after this run.	
38th day, wt 189 gm		54th day, wt 146 gm	
Time after test meal	R Q	Time after test meal	R Q
hrs		hrs	
3 5	1 06	3 5	1 09
4 5	1 04	4 5	1 02
5 5	1 05	5 5	1 02
6 5	1 02	6 5	1 00
Metabolic rate 4 4 calories		7 5	0 99
39th day, wt 186 gm		8 5	1 03
Time after test meal *	R Q	Metabolic rate 7 2 calories	
hrs.		55th day, wt 142 gm.	
5 5	1 05	Time after test meal *	R Q
6 2	1 01	hrs	
7 4	1 02	3 5	1 04
8 5	1 00	4 6	0 96
Metabolic rate 4 8 calories		5 7	1 01
40th-44th, 47th-50th days, dextrin, CaCO ₃ , NaCl ‡		6 7	0 99
46th day, wt 161 gm		Metabolic rate 6 0 calories.	
Time after test meal *	R Q	Died 56th day.	
hrs			
3 1	1 17		
4 1	1 08		
5 2	1 06		
6 3	1 00		
7 4	0 98		
Metabolic rate 4 9 calories			

* See under "Test Meals" in text

† Dextrin 40 parts, rolled oats 60 parts, CaCO₃ 2 parts, NaCl 2 parts.‡ Dextrin 95 parts, CaCO₃ 5 parts, NaCl 2 parts

TABLE III.
Rat on Restricted Diet and Later Summer Squash and Potato.

Rat 30. Began restricted diet Aug. 9; weight 130 gm.

1st-11th days, dextrin		33rd day, wt 105 gm.	
12th-20th, 23rd days, dextrin, rolled oats, CaCO ₃ , NaCl *		Time after test meal †	R Q
22nd day, wt 105 gm		hrs	
Time after test meal †	R Q		
hrs			
3 3	1 24	3 5	0 92
4 3	0 92	4 5	0 95
5 3	0 81	5 5	0 91
Metabolic rate 5 4 calories.		6 5	0 93
		7 5	0 94
		Metabolic rate 7 5 calories.	
25th day, wt 110 gm		35th day, wt 103 gm	
Time after test meal †	R Q	Time after test meal †	R Q
hrs		hrs	
3 8	0 90	3 6	0 95
4 8	0 95	4 6	1 00
5 9	1 45	5 7	0 98
7 1	1 64	6 8	0 97
8 1	2 03	7 9	1 09
Metabolic rate 5 0 calories		8 9	0 97
Summer squash, 55 gm, after this		9 9	0 98
run and during 26th day		Metabolic rate 5 2 calories.	
28th day, wt 112 gm		37th day, wt 99 gm.	
Time after test meal †	R Q	Time after test meal †	R Q
hrs		hrs	
2 6	0 80	2 6	0 87
3 6	0 63	3 6	0 97
4 7	0 92	4 6	0 95
5 9	1 00	5 6	0 95
7 2	0 91	6 6	0 95
8 3	0 87	7 6	0 95
9 3	0 93	Metabolic rate 5 5 calories.	
Metabolic rate 4 0 calories		38th day, wt 98 gm	
30th day, wt 106 gm		Time after test meal †	R Q
Time after test meal †	R Q	hrs	
hrs			
3 5	1 50	4 7	0 99
5 0	2 15	5 8	1 00
Potato, 20 gm, after this run and		6 8	1 07
during 31st day.		Metabolic rate 6 1 calories	
		Killed for blood sugar and glycogen	
		determinations.	

* Dextrin 40 parts, rolled oats 60 parts, CaCO₃ 2 parts, NaCl 2 parts.

† See under "Test Meals" in text.

small amounts of fats have an influence on the abnormal metabolism greatly in excess of their possible influence as fats *per se* (Table II, Rat 27, 31st and 36th days; Rat 28, 29th and 30th days; Rat 32, 54th and 55th days, Rat 44, 17th, 19th, and 20th days).

(c) Other fats in the same amounts have no influence in correcting the abnormal condition with regard to carbohydrates (Table II, Rat 26, 29th day, Rat 27, 29th day; Rat 28, 27th day).

4. High respiratory quotients were not obtained because of overfilling of the storage capacity of the animals for carbohydrate. It is worthy of note that in many instances the feeding of the test meal for 2 or more successive days did not result in high quotients, while in nearly every case in which extremely high quotients were obtained, a day or 2 of fasting preceded the test. Furthermore, a run made on Rat 10 (not shown in the tables) 24 hours after the third test meal on 3 successive days showed that an appreciable amount of carbohydrate was still being burned by the rat (RQ 0.92), but, at the end of the 32nd hour very little carbohydrate combustion was taking place (RQ 0.78). It is thought, for these reasons, that possible error due to the overfilling of the glycogen stores has been obviated in these experiments by the day or 2 of fasting which precedes most of the test meals.

SUMMARY.

1. Rats that were fed a restricted diet over a period of weeks were found to give respiratory quotients as high as 2 or more during the assimilation of a carbohydrate meal, indicating the conversion of a portion of the carbohydrate to fat.

2. When the restricted diet was modified so as to include casein, rolled oats, calcium carbonate, and sodium chloride, high respiratory quotients and thus probably fat formation were still obtained during the assimilation of a carbohydrate feeding.

3. If a small amount of lard or certain other hog fats were fed to rats which exhibited this abnormal conversion of carbohydrate to fat, normal assimilative respiratory quotients were obtained.

INDOLE DERIVATIVES IN CONNECTION WITH A DIET DEFICIENT IN TRYPTOPHANE.

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Various physiological data strongly suggest a possible close metabolic relation between an α -amino acid and a group of derivatives such as the corresponding acrylic, lactic, and pyruvic acids. Diets (generally fed to white rats as experimental animals) with the protein fraction distinctly deficient in any one amino acid indispensable to growth have permitted further investigation of this relation. McGinty, Lewis, and Marvel (1924-25) have found that *dl*- α -hydroxy- ϵ -aminocaproic acid among a group of similar derivatives does not replace lysine in a lysine-deficient diet. Cox and Rose (1926) have shown that imidazole acrylic acid as well as a number of other imidazoles closely related structurally to histidine do not replace this amino acid in a diet from which it has been removed. But these last authors were able to demonstrate that *dl*-imidazole lactic acid to a large extent does replace histidine. This was confirmed by Harrow and Sherwin (1926) who also found that imidazole pyruvic acid was available under the same conditions, but to a lesser extent.

It occurred to the writer that it would be of interest to prepare compounds closely related in structure to tryptophane, and then to incorporate these in a diet deficient in tryptophane. Sure (1925), after carrying out experiments to ascertain whether indole and alanine would condense to give tryptophane, came to the conclusion that they do not. This is in accord with the results of Cox and Rose (1926) that imidazole and 4-methylimidazole do not suffice in the place of histidine, and with the results of Lewis and Root (1920) that nor-leucine will not replace lysine. The carbon to hydrogen linkages indicated in these various instances do not

appear to be suitable for animal synthesis. Consequently, in the case of derivatives containing the indole ring intact, of special interest to the writer are those having position (3) side chains of the reactive type: 1 and 2 carbon aldehydes and the three 3 carbon acids mentioned at the beginning. In this connection, Jaffé and Cohn (1887) have reported that furfuraldehyde condenses with acetic acid in rabbits and dogs to give furfuracrylic acid. A similar reaction in the case of 3-indole aldehyde followed by addition of ammonia in the proper fashion would give tryptophane. The 3-indole acetaldehyde is very closely related to the corresponding pyruvic, lactic, and amino acids. Of particular importance is the β -3-indole lactic acid, since the corresponding histidine and lysine derivatives behave quite differently. For the preparation of these materials the literature affords a rather meager guide. The instability of the indole ring to some standard synthetic procedures undoubtedly accounts for this. However, 3-indole aldehyde and *l*- β -3-indole lactic acid have been prepared and their possible actions tested with a diet deficient in tryptophane.

EXPERIMENTAL.

For the preparation of a protein fraction deficient in tryptophane, advantage was taken of the instability of tryptophane toward mineral acid under the usual conditions of the acid hydrolysis of protein

1500 gm of technical casein were completely hydrolyzed by boiling with 5625 cc of water and 1875 cc of concentrated sulfuric acid on a sand bath for 18 hours in a 12 liter Pyrex flask fitted with a water-cooled reflux condenser. The cooled material was divided into halves. Each half was treated as follows: the liquor was diluted to about 15 liters and then neutralized by gradually stirring in powdered barium hydroxide (the hydrate). The barium sulfate was filtered off after the mixture was proved to have the very slightest excess of sulfuric acid.

The precipitate was twice washed with 5 liters of warm water. The combined filtrates amounted to about 20 liters. This volume was reduced to less than a liter in a vacuum still at a temperature between 40–60°C. The material was then dried in an air blast at a temperature between 50–90°C, and finally was ground to a powder that would pass a 40 mesh sieve.

The 3-indole aldehyde was prepared from indole by an application of the Grignard synthesis according to the directions of Majima and Kotake (1922). Indolyl magnesium iodide is prepared in anisole as the solvent. After the addition of the ethyl formate, the usual water-acid decomposition

is carried out. The 3-indole aldehyde is separated from the anisole and remaining indole, and then is crystallized from hot water. A yield of 2.2 gm of the aldehyde from 5.9 gm of the indole is recorded. The present writer secured from 5.9 gm of indole yields as follows: 1.0, 0.4, 1.4, and 1.1 gm. Majima and Kotake in their directions indefinitely emphasize cooling with ice and salt at the ethyl formate step. This was done but because of suspicion that the desired reaction might not be complete (persistence of deep red color), the reaction mixture was generally allowed to warm to room temperature with continued mechanical stirring for an hour. The poorest yield was secured when this was not done. Very recently Putochin (1926) has shown that heating to 70–75°C or even a higher temperature at the ethyl formate step does increase the yield to 2.2 gm or thereabouts per 5.9 gm of indole. Cooling sufficiently at this point according to him results in 1-formylindole exclusively.

The sample of 3-indole aldehyde used was white. It melted at 190–193°C. (corrected). For this compound Ellinger (1906) reports a melting point of 195°C, Majima and Kotake (1922) 193–195°C., and Putochin (1926) 194°C. The nitrogen by the Kjeldahl method amounted to 9.55 per cent as compared with the theoretical content of 9.65 per cent.

The *l*-β-3-indole lactic acid was prepared with a few modifications¹ according to the *Ordium lactis*² biological method of Ehrlich and Jacobsen (1911). A total of 6 gm. of tryptophane in three equal runs yielded 3.95 gm. of the desired product in the form of a very light brown crystalline material with an approximate melting point of 92–98°C. This was recrystallized from benzene to give 1.28 gm. of white crystals (Sample I). The remainder was purified by fractional precipitation from ether with petroleum ether to give 0.60 gm of white crystals (Sample II). Sample I melted at 100.0–101.0°C. (corrected), Sample II melted at 98.5–99.0°C. (corrected). A mixture of the two melted at 100°C (corrected). Ehrlich and Jacobsen (1911) record a melting point of 99°C. Titration with standard sodium hydroxide and phenolphthalein as the indicator gave a neutralization equivalent of 207.4. The calculated value is 205.1. The optical activity of a dilute water solution was determined in a Schmidt-Haensch saccharimeter³ graduated in

¹ Chief of these modifications, perhaps, was the employment of hot benzene as extracting agent at the semifinal stage. This permitted the same yield that Ehrlich and Jacobsen record at this point, but a much purer material. The ether-petroleum ether precipitation described by these authors is probably superior for removing the last traces of color in the purification process.

² Appreciation is here expressed to Prof. F. W. Tanner, of the Department of Bacteriology, University of Illinois, who kindly supplied a pure culture of *Ordium lactis*.

³ The instrument was generously made available by Prof. D. T. Englis of the Chemistry Department, University of Illinois.

Ventzke degrees and equipped with a tungsten ribbon lamp to provide the light which was passed through a bichromate cell.

$$[\alpha]_D^{20} = \frac{-0.46^\circ \times 20 \times 0.3468}{2 \times 0.2970} = -5.37^\circ.$$

It was found necessary to remove a very slight turbidity by means of "Filter-Cel," but no attempt was made to estimate any possible adsorption. Ehrlich and Jacobsen (1911) report for this substance $[\alpha]_D^{20} = -5.34^\circ$.

The methods employed in the rat feeding were essentially those

TABLE I

	Diet No		Diet No		
	206	207	207+a	207+b	207+c
Casein, technical, whole	18 0		100 gm	100 gm	100 gm
Casein digest		14 4	Diet 207	Diet 207	Diet 207
Cystine		0 3	+ 300 mg	+ 301 mg	+ 427 mg
Tyrosine		0 3	trypto-	<i>l</i> - β -3-	3-indole
Dextrin, white	36 0	39 0	phane	indole	aldehyde
Sucrose	15 0	15 0		lactic	
Salt mixture	4 0	4 0		acid	
Agar (ground)	2 0	2 0			
Lard	20 0	20 0			
Cod liver oil	5 0	5 0			

Vitamin B was supplied in daily pills containing each 300 mg of dextrin and 50 mg of Yeast Vitamine Harris. The amino acids used were prepared by standard methods and were analyzed to prove their purity. Cystine and tyrosine were added to compensate for possible losses in the protein digestion procedure. The salt mixture was prepared according to Osborne and Mendel (1919). The cod liver oil used was kindly supplied by the E. L. Patch Company, Boston.

described by Ferry (1919-20). Food consumption and body weight changes were recorded every 4 days. The young animals were permitted to demonstrate their growing capacity on Diet 206 (Table I), and then were transferred to Diet 207. This always resulted in continued distinct losses in body weight. Addition of 300 mg. of tryptophane per 100 gm. of Diet 207 caused an immediate and continued reversal of this effect. The 300 mg. of tryptophane judged by work of Furth and Lieben (1922) are probably more than are necessary for growth purposes, but for that reason

the required amount is well insured. In testing the *l*- β -3-indole lactic acid the equivalent of 300 mg. tryptophane was used, but in testing the 3-indole aldehyde, 2 equivalents were employed per 100 gm. of Diet 207.

The results of the investigation outlined in the preceding pages are presented in Charts I and II and Tables II and III. Invariably in these experiments, the food consumption decreased and increased with the respective omission and addition of tryptophane. It is evident from the continued smoothness of the growth curves that *l*- β -3-indole lactic acid and 3-indole aldehyde do not appreciably influence growth in the absence of tryptophane.

As it occurred to McGinty, Lewis, and Marvel (1924-25), so it also occurred to the present writer that failure of absorption might explain the inability of some likely derivatives to replace amino acids in the diet. Though certain related compounds might well be vitally connected with the intermediary metabolism of the amino acids after absorption, it does not follow that these derivatives would be selected for absorption from the alimentary tract with the same avidity as are the amino acids themselves native to that part of the protein assimilation scheme. Small amounts of the *l*- β -3-indole lactic acid were therefore injected subcutaneously over a short period, but there was no apparent increase in the body weights of the rats in question which were ingesting Diet 207. Similar injections of tryptophane under like conditions surprisingly brought the same results. This matter was followed up by allowing three rats to grow to over 150 gm. in weight so that they would be more suitable for injection work. A 36 day experiment was divided into three 12 day periods. During the first and last 12 days, the rats ingested deficient diet plus tryptophane, that is Diet 207 + a. Throughout the middle 12 day period they ingested only the deficient diet, Diet 207, but received injections of tryptophane in water (Régime 207+A) or just water (Régime 207+W) daily between 7 and 8 p.m. Reasonable care was exercised to prevent subsequent infection or other ill effect. The injections were made in two 0.5 cc. portions which were scattered over the dorsal and lateral surfaces of the rat bodies. The amount of 25 mg. of tryptophane per injection each day was based upon the quantity of tryptophane in the maximum daily food consumption in the fore period. It is obvious from Chart III that the

tryptophane requirement for growth injected rather than ingested did not allow the animals even to maintain their weights. Table IV makes it clear that a large and consistent variation in food

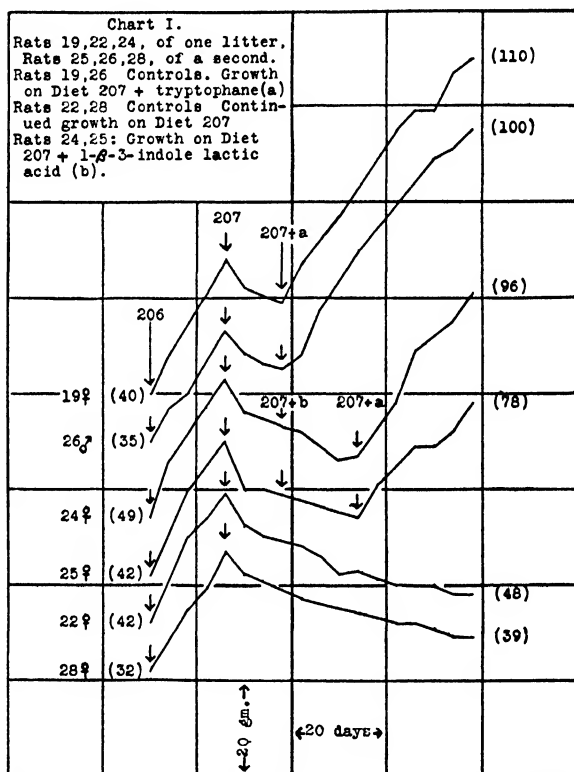


CHART I. All curves in any one chart are simultaneous. The arrows indicate dietary changes. The first number directly above an arrow denotes the diet introduced. Thus, for example, the dietary changes for Rats 24 and 25 in Chart I are identical. The initial and final body weights are shown in parentheses. Since the charts supplemented by the text are self-explanatory, the chart headings are designed to emphasize only the more important facts for consideration in connection with the growth curves.

consumption is not apparent as a possible explanation of these results. Further injection of tryptophane derivatives under parallel conditions was consequently abandoned.

It was further possible in the case of the lactic acid, that the enantiomorph of greater effect was not at hand. As far as the writer is aware, there is no basis for predicting correlation in this case between rotation and configuration, or further, for predicting the rotation or configuration that an animal organism would select if selection were made. The solution of this problem seemed to lie in racemization of the *l*- β -3-indole lactic acid.

Accordingly 1.7 gm. of partially decomposed residues of the

TABLE II.
Food Consumption

Days	Diet	Average daily food consumption	Diet	Average daily food consumption
Rat 19 ♀			Rat 26 ♂	
		<i>gm</i>		<i>gm</i>
1-16	206	4 2	206	3 7
17-28	207	3 2	207	2 7
29-44	207+a	4 4	207+a	4 1
45-68	207+a	5 3	207+a	4 5
Rat 24 ♀			Rat 25 ♀	
1-16	206	5 6	206	4 7
17-28	207	3 7	207	3 4
29-44	207+b	3 2	207+b	2 7
45-68	207+a	5 2	207+a	3 9
Rat 22 ♀			Rat 28 ♀	
1-16	206	5 0	206	3 4
17-28	207	3 6	207	3 2
29-44	207	3 8	207	2 6
45-68	207	3 1	207	2 4

lactic acid and 10 gm. of barium hydroxide (the hydrate) with 100 cc. of water were placed in a 200 cc. round bottom flask fitted with a reflux condenser. Boiling at a temperature of about 100°C. was continued for 10 hours. The process then was: cooling, acidification with sulfuric acid, filtering off of the barium sulfate, extraction of the filtrate with ether, evaporation of the ether, crystallization of the residue from benzene. 0.14 gm. of pure white crystalline material was obtained. In water solution, this exhibited an acid reaction, and gave a positive Hopkins-Cole test

as did, in fact, all the indole compounds experimentally considered in this paper. The melting point was 98–100°C. (corrected). There was no doubt that the recovered substance was indole lactic acid. Owing both to the small amount of material and lack of facilities at the time, measurements of the optical activity were

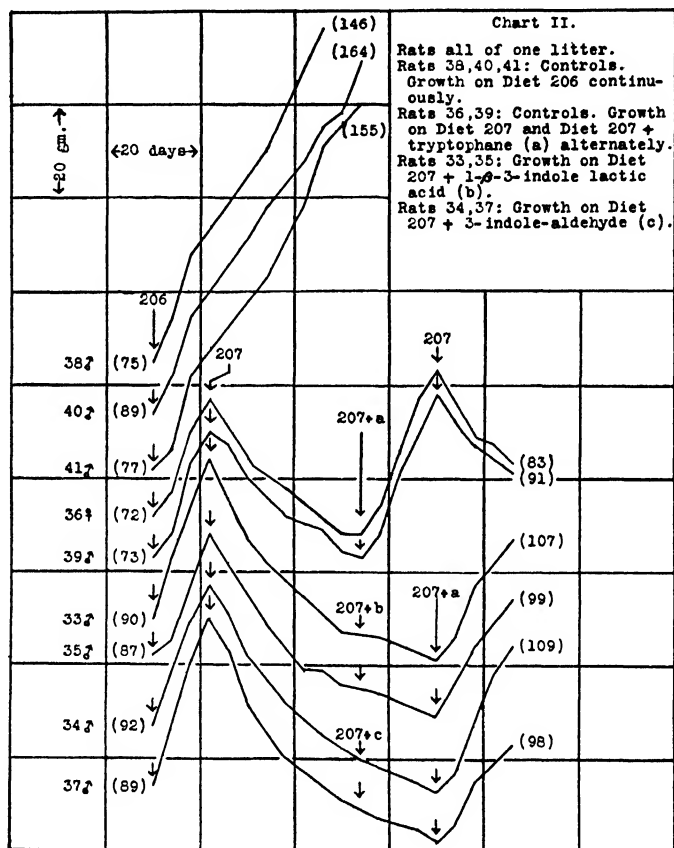


CHART II.

not undertaken. A melting point of a mixture of this presumably racemized β -3-indole lactic acid and of Sample II of the aforementioned *l*- β -3-indole lactic acid showed no deviation from the melting points of the two original materials. A second and larger sample but somewhat less pure than the first was also isolated.

Feeding of the material for 8 days to a rat on Diet 207 caused not the smallest change in a very smooth growth curve, notwithstanding that, before and afterwards, introduction of tryptophane resulted in very distinct body weight additions within 24 hours. The writer believes that even this brief experiment constitutes very strong evidence that *dl*- β -3-indole lactic acid is not available to replace tryptophane in the absence of the latter. This would mean that the tryptophane and lysine hydroxy deriva-

TABLE III
Food Consumption.

Days.	Diet	Average daily food consumption	Diet	Average daily food consumption	Diet	Average daily food consumption	Diet.	Average daily food consumption.
	Rat 40 ♂		Rat 41 ♂		Rat 36 ♀		Rat 39 ♂	
		gm		gm		gm		gm
1-12	206	7 9	206	7 3	206	7 0	206	6 7
13-28	206	7 2	206	6 5	207	4 3	207	5 2
29-44	206	7 8	206	7 8	207	3 7	207	4 0
45-60					207+a	7 1	207+a	6 3
61-76					207	4 8	207	5 3
	Rat 33 ♂		Rat 35 ♂		Rat 34 ♀		Rat 37 ♂	
1-12	206	8 3	206	6 8	206	8 2	206	8 5
13-28	207	5 8	207	4 6	207	5 1	207	4 6
29-44	207	4 4	207	3 8	207	4 2	207	3 6
45-60	207+b	3 9	207+b	3 8	207+c	3 6	207+c	3 6
61-76	207+a	5 6	207+a	4 6	207+a	5 1	207+a	4 9

tives behave similarly and differently from the hydroxy derivative of histidine.

SUMMARY.⁴

1. A diet deficient in tryptophane and therefore suitable to certain studies of the intermediary metabolism of this indispensable amino acid has been described.

⁴ After preparing the manuscript for this article, the author observed that on p. 155 of the 9th edition of Hawk and Bergem's "Practical Physiological Chemistry" brief mention is made of unpublished work of Heft and Sherwin to the effect that neither indole pyruvic acid, indole lactic acid, nor kynurenic acid can replace tryptophane in the diet.

2. The growth of white rats upon this diet deficient in tryptophane was not appreciably influenced by the addition of either 3-indole aldehyde or *l*- β -3-indole lactic acid.

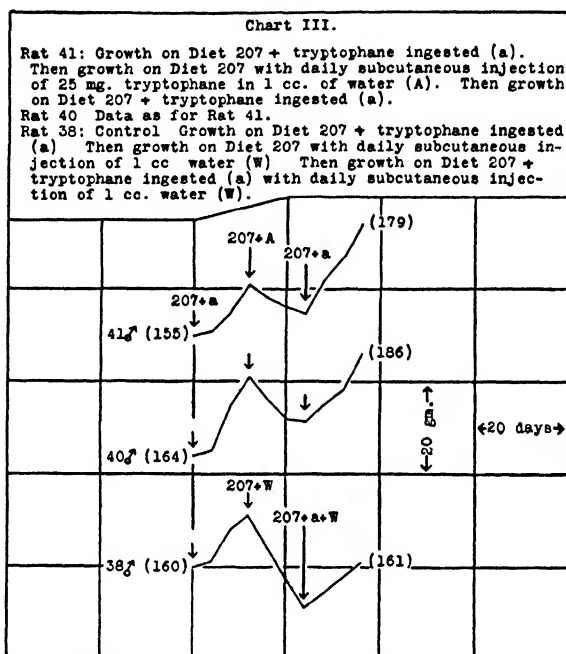


CHART III.

TABLE IV
Food Consumption.

Days	Diet or régime	Average daily food consumption	Diet or régime	Average daily food consumption	Diet or régime	Average daily food consumption
	Rat 41 ♂		Rat 40 ♂		Rat 38 ♂	
		gm		gm		gm
1-12	207+a	7 5	207+a	9 0	207+a	7 4
13-24	207+A	7 3	207+A	8 1	207+W	6 3
25-36	207+a	7 8	207+a	7 6	207+a+W	5 4

3. The orally administered tryptophane requirement for growth has been shown not to be sufficient even to maintain body weight when injected subcutaneously over 12 day periods.

4. A sample of the active lactic acid subjected to the probable racemizing action of long boiling in barium hydroxide solution exhibited no appreciable effect on growth in connection with the diet deficient in tryptophane. This evidence points to the conclusion that the α -hydroxy derivative of tryptophane behaves similarly to that of lysine and differently from that of histidine.

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THE METABOLISM OF SULFUR.

XII. THE VALUE OF DIGLYCYL-CYSTINE, DIALANYL-CYSTINE, AND DIALANYL-CYSTINE DIANHYDRIDE FOR THE NUTRI- TIVE REQUIREMENTS OF THE WHITE RAT.

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Studies of the physiological behavior of the peptides and their derivatives are not of frequent occurrence in the literature of biochemistry. Particularly is this true of the peptides, in whose molecule the amino acid cystine is contained. Interest in these compounds has been greatly stimulated by the discovery of glutathione (1), a peptide of cystine or cysteine and glutamic acid, as well as by the recent studies of the sulfur content of insulin (2) and of the change in the stability of the cystine sulfur in peptides and piperazines containing this amino acid by Bergmann and Stather (3) and by Brand and Sandberg (4).

In this laboratory we have been interested in the physiological behavior of organic compounds containing sulfur, particularly derivatives of cystine (5-8), and have been able to demonstrate that by protecting the amino group of cystine so that deamination is prevented, the oxidation of the sulfur fraction of cystine does not follow its normal course (5, 7). We have also observed that, although taurine is probably derived from cystine, taurine cannot replace cystine for purposes of growth in the young white rat (8), even under conditions of an urgent demand for cystine, a conclusion which was reached also by Rose and Huddlestun (9). In the present study, we have investigated the utilization for purposes of growth by the young white rat of two cystine-containing peptides, diglycyl-cystine and dialanyl-cystine, and of the 2, 5-diketopiperazine derived from dialanyl-cystine, dialanyl-cystine dianhydride. The behavior of this last compound has

seemed of unusual interest in view of the possible occurrence of piperazine rings in the protein molecule and of the marked lability of the cystine sulfur in this particular anhydride (3, 4).

Pepsin failed to split any of the synthetic peptides prepared by Fischer. Included in the list of those studied (10) were dialanyl-cystine and dileucyl-cystine, which were, however, readily hydrolyzed by the pancreatic juice of the dog. Glutathione (glutamyl-cysteine), on the other hand, is probably able to function in the organism because of its resistance to hydrolysis by enzymes and to other like metabolic changes (1). The action of the enzymes of the alimentary tract on diglycyl-cystine has not been investigated. Abderhalden and Samuely (11) have compared the absorption and oxidation of cystine and cystine in combination as a dipeptide, dialanyl-cystine or dileucyl-cystine, in experiments on dogs. If cystine was fed by mouth, 80 to 95 per cent of the administered sulfur was eliminated within 48 hours by the kidneys, for the most part as oxidized sulfur. When an equivalent amount of cystine was fed as dialanyl-cystine, 70 per cent of the ingested sulfur appeared in the urine within the same period of time. With dileucyl-cystine, the recovery of sulfur in the urine was slightly lower (64 per cent). When the dipeptides were injected subcutaneously as the sodium salts, the percentages of injected sulfur appearing in the urine within 48 hours were approximately the same (73.3 and 63.7 per cent for dialanyl-cystine and 63.8 per cent for dileucyl-cystine). In the dog, dialanyl-cystine was absorbed, either before or after hydrolysis, and the sulfur was metabolized to nearly the same extent as when pure cystine was fed. Even though dialanyl-cystine is not readily soluble, absorption was not at fault, since the same recovery of extra sulfur was obtained after subcutaneous injection as after oral administration.

The fate of the anhydrides of peptides (2, 5-diketopiperazines) in the animal organism has been studied by Abderhalden and collaborators (12-14). The behavior of these compounds, when fed by mouth, appears to vary with the nature of the amino acids present and to some extent with the individual animal. In some cases, the anhydride was excreted unchanged in the urine for the most part; in others, it was hydrolyzed and the resultant amino acids or peptides, either excreted by the kidneys, metabolized, or stored. In all experiments, the compounds appeared to

be readily absorbed from the alimentary canal, despite their marked insolubility in water. Brand and Sandberg (4) injected intravenously and intraperitoneally the anhydride of dialanil-cystine into fasting rabbits and observed no toxic effects nor influence on blood sugar concentration. The amounts injected were, however, very small (about 13 and 60 mg. per kilo).

Our experiments have given evidence that diglycyl-cystine and dialanil-cystine are as well utilized by the white rat for growth as an equivalent amount of cystine. When the anhydride of dialanil-cystine was fed, however, no increased rate of growth as compared with that of the rats on the basal cystine-deficient diet was noted.

TABLE I
Composition of Diets.

	Basal.	Diet G	Diet H	Diet I	Diet J
	gm.	gm.	gm	gm.	gm.
Casein	9 0	9 0	9 0	9 0	9 0
Salt mixture	4 5	4 5	4 5	4 5	4 5
Corn-starch.	54 5	54 5	54 5	54 5	54 5
Sucrose	4 5	4 5	4 5	4 5	4 5
Lard	24 5	24 5	24 5	24 5	24 5
Cod liver oil.	3 0	3 0	3 0	3 0	3 0
Cystine.		0 34			
Diglycyl-cystine			0.50		
Dialanil-cystine.				0 54	
“ dianhydride.					0 49

EXPERIMENTAL.

The diglycyl-cystine used in the experiments was prepared in this laboratory¹ by the method of Abderhalden and Spinner (15). Dr. Erwin Brand of the Montefiore Hospital, New York City, very kindly placed at our disposal the dialanil-cystine and its dianhydride. The methods used in their preparation have already been described by him (4).

The general procedure of our animal experiments was the same as that previously reported (8). Young white rats of 30 to 50 gm. in weight were placed upon the basal cystine-deficient diet, the

¹ We are indebted to R. H. Wilson and F. H. Wiley for the preparation of the diglycyl-cystine.

basal diet plus cystine, and the basal diet plus the peptide or anhydride to be studied. In every case litter units were used, the litter mates serving as controls on cystine-deficient and cystine-containing diets. The composition of the diets is given in Table I. The amounts of the peptides, anhydride, and cystine were so adjusted that the diets contained equivalent amounts of cystine. The casein was a commercial product (Harris casein, free from vitamin B); the salt mixture was that of Osborne and Mendel. Water-soluble vitamin was supplied by Vegex, a commercial yeast concentrate

DISCUSSION.

The results of the experiments, which comprised a series of five litters of rats, are presented in Table II.

It will be noted that the animals of Litters 13 and 21, which received the diglycyl-cystine, showed almost exactly the same increase in weight as did their litter mates whose diet contained an equivalent amount of cystine in place of the peptide. The rats of Litter 15, which were fed the peptide, did not make quite so great a gain in weight during the experimental period as did the animal which was fed cystine. In view of the results with the other litters, we do not feel that this difference is significant, especially since there is a very definite increase in the rate of growth over that of the animals of this litter, which received the basic cystine-deficient diet.

The results with dialanyl-cystine, although few in number, were equally definite and uniform. The two animals to which this compound was fed (Rats 191 and 195) during a period of 3 weeks showed very nearly the same increase in weight as did their litter mates maintained during the same period on the basal diet with the addition of an amount of cystine equivalent to the cystine content of the peptide.

Our experiments with dialanyl-cystine dianhydride were not quite so definite. Due to the small amount of the dianhydride available for our experiments we were not able to continue our feeding studies longer than 2 weeks with one rat (No. 192) and 3 weeks with another (No. 194). Rat 192 did not eat well while on the diet containing the dianhydride, its average daily food consumption for the 2 weeks being 4.9 and 4.5 gm. only, as compared

with an average daily intake of approximately 6 gm. of food for the control rats of the same age and weight. It may be noted, however, that Rat 126, during the 2nd week of the experimental

TABLE II

Litter No	Rat No	Duration	Diet	Initial weight	Final weight	Gain
		<i>wks</i>		<i>gm</i>	<i>gm</i>	<i>gm</i>
13	113	5	Basal and diglycyl-cystine.	55 5	129 5	74 0
	114	5	" " "	55 0	122 0	67 0
	115	5	Basal.	56 0	100 5	44 5
	116	5	"	61 0	100 5	39 5
	117	5	" and cystine.	50 5	123 0	72 5
15	124	5½	Basal and diglycyl-cystine	50 5	111 0	60 5
	125	5½	" " "	51 0	115 0	64 0
	126	5½	Basal	54 0	94 0	40 0
	127	5½	"	55 0	97 5	42 5
	128	5½	" and cystine	55 0	139 5	84 5
21	179	6	Basal.	44 5	94 5	50 0
	180	6	"	41 5	93 0	51 5
	181	7	" and diglycyl-cystine.	43 5	116 0	72 5
	182	6	" " "	44 0	128 5	84 5
	183	6	Basal and cystine.	39 0	121 0	82 0
	184	6	" " "	37 5	128 0	90 5
22	187	5	Basal	54 5	96 5	42 0
	188	3	"	58 0	77 5	19 5
	189	5	" and cystine	55 0	140 5	85 5
	190	3	" " "	58 5	107 5	49 0
	191	3	" " dialanyl-cystine.	45 0	93 0	48 0
	192	2	" " anhydride	47 0	47 0	0 0
		1	" " dialanyl-cystine.	47 0	67 5	20 5
		2	" " cystine	67 5	100 0	32 5
23	193	4	Basal and cystine	50 5	110 0	59 5
	194	3	" " anhydride.	49 5	65 0	15 5
		1	" " dialanyl-cystine.	65 0	88 5	23 5
	195	3	" " "	48 0	92 5	44 5
	197	4	"	48 5	84 0	35 5

period, had an average daily food intake of only 4.9 gm., and was able, nevertheless, to maintain its body weight. Furthermore, this animal weighed 66 gm. while Rat 192 during the period under discussion weighed about 47 gm. and its food requirements for

maintenance must have been considerably less than those of Rat 126. Similarly, Rat 124, with an average daily food intake of 5.0 and 4.7 gm. during 2 successive weeks, gained 5 and 4.5 gm. respectively. Numerous other instances of the same kind might be cited from the records of the food intake, and hence it seems hardly probable that failure to grow was due entirely to the small amount of food eaten.

In the case of Rat 194, the food intake was greater and the animal showed a slow gain in weight while fed the diet containing the dianhydride. This increase, however, was only a little more than half that made by the control littermate on the 9 percent casein diet without added cystine. During the last week of the 3 week period of the experiment, the two animals gained exactly the same amount, 10.5 gm. It seems reasonable to suppose that, if it had been possible to continue the experiment over a longer period of time, the rat on the diet containing the dianhydride would have shown a rate of growth similar to the control on the 9 per cent casein diet.

Both Rat 192 and Rat 194 were maintained for 1 week on a diet containing dialanyl-cystine after the feeding of the dianhydride was discontinued. In each case, a markedly increased rate of growth resulted. Rat 192 gained 20.5 gm. in 1 week and continued to gain at this rapid rate in the 2 following weeks on a cystine diet, so that its failure to show satisfactory growth on the anhydride diet was not due to lack of ability to grow when given an adequate diet. Similarly Rat 194, which gained 10.5 gm. during the 3rd week on a diet containing the dianhydride, made a gain of more than double this amount (23.5 gm.) in a week, when placed on a diet containing the dialanyl-cystine.

Among the possible causes of the failure of the dialanyl-cystine dianhydride to serve as an available source of cystine, the question of its absorption from the alimentary canal must be considered. As previously noted, Abderhalden and his collaborators (12-14) found that the anhydrides, which they studied, although very sparingly soluble, were readily absorbed. We have made use of the marked lability of the sulfur in dialanyl-cystine dianhydride (3, 4) in an attempt to determine whether failure of absorption of this substance was responsible for the results we have obtained. The sulfur of this compound is readily split by dilute alkali, even in the cold, whereas the peptide is less readily attacked and the

sulfur of the cystine is rather stable under these conditions. If, then, the dianhydride was not absorbed, but was excreted in the feces, the feces of a rat on a diet containing the dianhydride should contain significant amounts of labile sulfur. Feces were collected for a period of a week from three rats, one fed cystine, one dialanyl-cystine, and one the dianhydride in addition to the basal diet. 1 gm. of each of the three samples of feces was heated in a boiling water bath for 15 minutes with 10 cc. of 10 per cent sodium hydroxide. The tubes were cooled, neutralized, and acidified with hydrochloric acid. Strips of filter paper moistened with lead acetate solution were suspended in the mouth of the tubes, which were then tightly stoppered. On standing for a few minutes, hydrogen sulfide in appreciable amount was liberated but no differences could be observed in the evolution of hydrogen sulfide as evidenced by the blackening of the lead acetate paper.

Since it seemed possible that this experiment might not be sufficiently delicate, we have carried out a roughly quantitative experiment. 0.5 gm. portions of the same samples of feces were finely pulverized, placed in test-tubes with 10 cc. of N sodium hydroxide, and allowed to stand at room temperature in contact with the alkali for 30 minutes. According to Brand and Sandberg (4), the sulfur of the dianhydride is readily split off under these conditions, while the sulfur of the corresponding dipeptide and of cystine is not greatly affected. The contents of each were filtered off and to the clear but rather deeply colored filtrates 10 drops of saturated lead acetate were added. The tubes were stoppered and allowed to stand for 24 hours. On the addition of the lead acetate to the filtrate from the feces of the animal which was fed the dianhydride, the development of the blackening was more rapid than with the other filtrates, and a decided precipitate was noted. After 24 hours, all three tubes contained a precipitate. The precipitates were filtered off in weighed Gooch crucibles and dried to constant weight at 100°. The weights of the lead sulfide were 5.6 mg. (cystine diet), 10.0 mg. (anhydride diet), and 7.7 mg. (peptide diet). For the weekly period, this would represent an excretion through the feces of 3.0, 5.4., and 4.1 mg. of labile sulfur respectively. Inasmuch as the sulfur intake (excluding the sulfur of the casein and salt mixture) was about 35, 36, and 49 mg. of sulfur respectively during the same period, this represents an unimportant loss of sulfur. When one considers the greater degree

of lability of the sulfur of the dianhydride as compared with the peptide or cystine itself, it seems impossible that lack of absorption is responsible for our negative results with the dianhydride, and that some other explanation, possibly inability to cleave the piperazine linkage,² must be sought. We cannot, of course, exclude the possibility that the dianhydride may be changed in its passage through the alimentary canal so that its sulfur is in a less labile combination. This seems hardly probable, however.

SUMMARY.

Experiments are described in which diglycyl-cystine, dialanyl-cystine, and the dialanyl-cystine dianhydride were fed to growing white rats maintained on a diet deficient in its content of cystine. Evidence was obtained that diglycyl-cystine and dialanyl-cystine were able to promote growth in the absence of an adequate supply of cystine. There were no indications that the dianhydride of dialanyl-cystine could be utilized for growth under these conditions. It is not believed that the unsatisfactory growth on the dianhydride is to be ascribed to failure of absorption of this constituent of the diet from the alimentary canal.

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² It is generally accepted that the diketopiperazine ring is not split by pepsin or trypsin. Levene and Pfaltz (16) recently stated that erepsin is also unable to effect this hydrolysis.

THE CYSTINE CONTENT OF HAIR AND OTHER EPIDERMAL TISSUES.

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There have appeared in the literature of recent years occasional reports concerned with the relation of cystine to the growth of hair and wool. Evvard, Dox, and Guernsey (1) in experiments with sows, and Sjollem (2), with rabbits, have suggested that the feeding of cystine or proteins high in their content of this amino acid was a factor in promoting an increased growth of hair. Certain German investigators (3, 4), who employed a commercial product (Humagsolan), a hydrolysate of keratin, reported similar results, which were not, however, confirmed by Fuhs (5). In connection with a study of factors concerned in the growth of hair in progress in this laboratory, we have had occasion to review the data concerning the sulfur and cystine content of the more common types of keratins. The data are open to the criticism that the degree of purification of the keratins has varied in the hands of different observers, no large comparative series, in which the methods of preliminary treatment and determination of cystine have been uniform, being available. We have accordingly considered it necessary for our purposes to determine by a uniform procedure the cystine and sulfur content of a considerable number of keratins. The results, while they may not be of absolute accuracy in view of the lack of an entirely satisfactory method for the determination of cystine, afford a more extensive comparative study of the keratins than has heretofore been available.

The cystine content of the keratins has usually been determined by isolation of the impure cystine from the protein hydrolysate and subsequent determination of the sulfur content of this

impure cystine. From the sulfur content, the cystine has been calculated. This method must give low results, since cystine, although precipitated almost quantitatively from pure solutions, is not precipitated completely in the presence of organic substances, such as would occur in a protein hydrolysate. We have shown previously (6) that this is true when cystine is added to urine, and will present in the present communication further evidence of the presence of cystine in the filtrate, after the precipitation of cystine from protein hydrolysates by the usual methods. A few determinations of the cystine content of keratins are available from the analyses by the Van Slyke method (7) of characterization of proteins by the determination of specific groups, the cystine being calculated from the sulfur content of the solution of the bases precipitated by phosphotungstic acid. Sources of error are a considerable correction for the solubility of the cystine phosphotungstate, as well as an alteration of a portion of the cystine by prolonged boiling with acid so that it is no longer precipitable by phosphotungstic acid (8). Van Slyke has estimated that the amount of cystine obtained by this method represents less than half of that actually present in the protein.¹ Analyses by the method of Folin and Looney, which we have employed in the present series, have been relatively few. This method, which depends upon the fact that, while cystine itself does not react with the uric acid reagent of Folin and Denis, in the presence of sodium sulfite it is reduced to cysteine, which gives a deep blue color with the reagent, has been criticized by Okuda (9) on the basis of non-specificity, since in his experiments chemical groups present in proteins, other than cystine, gave the same color as cystine. No further details are cited in the publications available to us, nor have we any experience with the iodometric method proposed by him (9).

In Table I are summarized the analytical data available for the cystine content of the tissues studied in the present investigation. Unless otherwise noted, the cystine determinations have been made by the older method which involves precipitation of the crude cystine from the hydrolysate and calculation of the cystine from the total sulfur content of the precipitate.

¹ Van Slyke (7), p 28-29

EXPERIMENTAL.

The keratins used in these experiments had been thoroughly extracted with organic solvents by Dr. Henry C. Eckstein, who has reported a study of their lipid content. The extraction was in four steps over a total period of 96 hours: treatment with boiling vapors of absolute alcohol, chloroform, ether, and again with absolute

TABLE I
Cystine Content of Epithelial Tissues

Source	Cystine content	Observer
	<i>per cent</i>	
Hair, human	6.5-13.92	Morner (10).
" "	11.5-14.53	Buchtala (11).
" "	9.10-9.90*	Sammartino (12)
" "	16.50†	Folin and Looney (13).
" "	13.2-17.4†	Klinke (14)
Wool, sheep	6.9-12.5	Abderhalden and Voitnikovici (15)
" "	7.8†	Folin and Looney (13).
Goose feathers (quills)	6.30	Buchtala (16)
Hair, dog	8.49*	Van Slyke (7)
Tortoise shell (<i>Chelone imbricata</i>)	5.19	Buchtala (17)

* Value calculated from the analysis by the Van Slyke method (7).

† Folin and Looney colorimetric method

alcohol (18). The keratins were then dried for several days at 100° and placed in tightly stoppered bottles.

1 gm. of the sample was usually used for the analysis. It was found advantageous, particularly in the case of the rabbit and rat hair and the fan portion of the feathers, to use a weighing tube, open at each end, about 1 cm. in diameter, into which the sample was packed as tightly as possible. After weighing, the sample could be transferred to the flask, in which the hydrolysis was carried out easily and without loss by pushing it out of the tube with a glass rod. The hydrolysates were tested by the biuret test, which was uniformly negative except in two cases, in which a faintly positive test was obtained.

Total sulfur was determined by the Denis modification of the Benedict method. Trotman and Bell (19), who made a comparative study of the methods of sulfur analysis in wool, consider this

method satisfactory. The hair or other tissue was heated over a low flame with 20 cc. of concentrated nitric acid in a long necked 500 cc. Kjeldahl flask for 4 hours to dissolve the material and permit of partial oxidation. Evaporation was prevented by inserting an inverted 50 cc. Erlenmeyer flask in the mouth of the Kjeldahl flask. The solution was made up to a volume of 105 cc. and aliquots of 50 cc. were used for the sulfur determination. These were evaporated to dryness in small evaporating dishes, about 20 cc. of water were added, and the solutions were again evaporated to dryness. This treatment was to remove the excess acid as com-

TABLE II
Cystine Content of Hair and Wool of Various Species

Source	No of samples analyzed	Cystine	
		Range	Average
		<i>per cent</i>	<i>per cent</i>
Human			
Children (1½-5 yrs)	7	15 6-19 9	18 7
" (7 yrs)	5	18 0-19 4	18 9
" (9-10 yrs)	5	16 5-21 2	19 4
" (12-13 ")	6	15 6-18 4	17 3
" (All ages)	23	15 6-21 2	18 9
Adult	6	16 8-18 4	17 6
Rabbit	4	11 9-14 0	13 0
Rat, white (normal)	1	14 1	14 1
" " (rachitic)	1	14 5	14 5
Cat	1	13 1	13 1
Dog	1	19 0	19 0
Sheep, wool	9	8 0-10 9	9 5

pletely as possible. The subsequent procedure was the same as in the sulfur determination by the Benedict-Denis method, 5 cc of the reagent usually being required for the oxidation. Nitrogen was determined by the Kjeldahl method.

DISCUSSION.

In Table II are summarized the results of the analyses of twenty-three samples of the hair of children of various ages. Frequently the sample obtained was so small that it was not possible to determine total nitrogen and sulfur in addition to the cystine. The

cystine values showed considerable variation, ranging from the minimum figure of 15.6 to a maximum of 21.2 per cent. The content of nitrogen was more constant than the cystine in all cases, varying only within narrow limits (15.25 to 15.50 per cent). The nitrogen to cystine sulfur ratio (2.7 to 3.2) and nitrogen to sulfur (3.0 to 3.2) ratio were relatively constant and approximated the ratios found for human hair by Rutherford and Hawk (20).

The high cystine figures obtained are striking, three samples containing more than 20 per cent of cystine. Two samples showed a cystine content as low as 15.6 per cent, but none approached the lower values (13.2, 13.3, and 14.5 per cent) found by Klinke (14). There seems to be no relation between the cystine content of the hair and the color of the hair, age, or sex. The nitrogen to sulfur ratios also fail to show any significant variations, so that while the ratios are similar to those of Rutherford and Hawk, it does not seem possible to draw the conclusions they suggest. Despite the fact that no treatment with gastric juice or any other enzyme preparation was made in our experiments, it may be noted that the nitrogen content of the hair of our series compared well with the values of Rutherford and Hawk, in whose experiments the hairs were treated successively with artificial gastric and pancreatic juice.

The values obtained for the hair of adults (Table II) also fail to demonstrate any of the above mentioned relationships. As a rule, the cystine sulfur was nearly equal to the total sulfur. Two specimens of adult red hair did not differ materially from the hairs of other color in their content of sulfur (5.05, 5.12 per cent, as compared with a range of 4.32 to 5.08 per cent and an average value of 4.84 per cent for hair of other colors), nor was the proportion of total sulfur not cystine sulfur as marked as in the red hair studied by Klinke (14). Although we recognize the dangers of reasoning from too small a series, we cannot fail to be impressed by the apparent tendency of the values for cystine in adult hair to be slightly lower than those in children's hair.

In Table II, the results obtained with four samples of rabbit hair are listed. The cystine values ranged from 11.9 to 14.0 per cent. The number of samples is too small to permit of consideration of individual variations, but is large enough to show that rabbit hair contains a greater amount of cystine than either wool

or feathers, the content of cystine approaching that of human hair. The samples of rat hair were mixtures of hair from several animals. There was no significant difference between the two samples, although one was from rachitic rats.² These samples and that from a silver Persian cat had a cystine content resembling that of rabbit hair. The one sample of dog hair analyzed is interesting since it contained as much cystine as human hair, a value much greater than that obtained by Van Slyke's partition method (7).

Considerable difficulty was experienced in the analyses of wool (Table II), due to the presence of dirt in the wool, which, even though removed as completely as possible, interfered with the determinations somewhat. Of the nine samples, only one approached the low value of 7.8 per cent of Folin and Looney (13) and none approximated the higher value of 12.5 per cent of Abderhalden and Voitinovic (15).

The scutes of several species of turtles³ were analyzed (Table III). The cystine contents of these keratins was lower than that of any of the types previously studied. The value obtained by Mulder (21) for sulfur (2.22 per cent) is comparable to the two values for total sulfur found in our series.

The most striking feature of the analyses of feathers (Table III) was the high cystine content of the fan portions of the goose feathers (10.7 per cent) and especially of the duck feathers (12.2 per cent). The other values were fairly uniform. The variation in the nitrogen to sulfur ratios was greater in this case than in any of the other keratins studied.

The three samples of skin analyzed (Table III) were all low in their cystine content and resembled the common types of proteins (22) in this respect more than the keratins. These values and that found for a sample of connective tissue (the residue obtained after extraction of the lipids from human adipose tissue) agreed fairly closely. Although the variations in the percentages of cystine and nitrogen were considerable, the ratios of nitrogen to cystine sulfur were very constant.

² We are indebted to Prof. Amy L. Daniels of the University of Iowa for the samples of hair of rachitic rats.

³ For the samples of tortoise shell and the identification of species we are indebted to Prof. A. G. Ruthven of the University Museum.

In general, throughout the series of analyses, the differences between the cystine sulfur and total sulfur were not large, and can usually be explained by experimental error. In the few cases where the difference was more marked, we may have indications of the existence of sulfur compounds other than cystine. In only two instances was there obtained a value for cystine sulfur which

TABLE III
Cystine Content of Tortoise Shell, Feathers, and Skin

Material	Cystine	Total sulfur	Nitrogen.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Tortoise shell			
<i>Pseudemys elegans</i>	7 0		
<i>Graptemys pseudogeographica</i>	7 5		
<i>Chrysemys marginata</i>	7 3		
<i>Testudo pardalis</i>	6 4		15 1
<i>Kinosternon odoratum</i>	7 9		
<i>Chelydra serpentina</i>	7 3	2 12	14 4
<i>Emys blandingii</i>	8 1	2 44	15 3
Feathers			
Turkey 1 Fan	7 1	2 44	15 1
Quill	8 9	2 40	15 8
Turkey 2 Fan	7 7		14 8
Quill	7 7		15 5
Goose Fan	10 7	3 00	15 1
Quill	9 1	2 53	
Duck Fan	12 2	2 90	15 5
Quill	8 8		15 9
Skin, human			
No 1	1 8	0 70	11 3
" 2	2 1		12 4
" 3.	2 3		15 3
Connective tissue *	2 3		15 0

* The residue from human adipose tissue after extraction with fat solvents.

was considerably in excess of the value for total sulfur. It is not our purpose in this paper to discuss the question of the types of sulfur compounds present in the keratins, but we believe there is little evidence to be obtained from our data in support of the contention that in these proteins there exists any considerable amount of non-cystine sulfur. The high cystine values obtained

by us cannot be due to the presence of sulfides, or mercapto compounds, such as thiolactic acid, since we have tested the reaction of our hydrolysates with the Folin-Denis uric acid reagent without the addition of sodium sulfite and have never observed a reduction of the reagent in the absence of the sulfite, which could influence significantly our colorimetric readings in the cystine determination.

We have also made a comparative study of the results of the Folin-Looney method and the older isolation method for cystine. 7 gm. of rabbit hair (Rabbit 177) were dissolved on the steam bath in a solution of 25 cc of hydrochloric acid (sp. gr. 1.18) and the same volume of water, and the heating was continued for 100 hours, after which the biuret test was negative. The hydrolysate was transferred quantitatively to a flask, and the combined hydrolysate and washings were evaporated to 50 cc under diminished pressure. The liquid was cooled, made slightly alkaline with 50 per cent sodium hydroxide and immediately 3 cc of glacial acetic acid were added. An equal volume of acetone was added to increase the insolubility of the cystine. The mixture was placed in the ice box for 2 days, the precipitate filtered off, and the filtrate allowed to stand in the cold. After several days, an additional precipitate appeared, which was also removed by filtration. A small amount of material was precipitated during the next week. The combined weight of the precipitates after drying was 1.012 gm. They were dissolved in dilute hydrochloric acid and the cystine content was determined colorimetrically according to Folin and Looney. The three precipitates contained 403, 190, and less than 1 mg. of cystine, or a total amount equal to 8.5 per cent of the original hair. The sulfur contents of the first two precipitates were then determined (the usual procedure in gravimetric cystine determinations) and found to be equivalent to 402 and 183 mg. of cystine, respectively, a total sulfur content which corresponded to 8.4 per cent of cystine in the hair.

The acetone in the filtrate after the precipitation of the crude cystine was removed by distillation, and the cystine content of the filtrate as determined colorimetrically was found to be 266 mg. or 3.8 per cent of the original hair. This figure added to the value calculated from the sulfur content of the crude precipitated cystine gives a value of 12.2 per cent of cystine in comparison with 12.3 per cent as determined directly by the Folin-Looney method.

The sulfur content of the filtrate was 80 mg., a value corresponding to 300 mg. of cystine, or somewhat more than that found colorimetrically.

A summation of the sulfur content of the precipitate and filtrate gives a total of 0.2364 gm. of sulfur, or 3.38 per cent of the original hair. The value as determined directly was 3.88 per cent.

A similar experiment was carried out with human hair. 23 gm. of hair were hydrolyzed with 75 cc. of concentrated hydrochloric acid and 75 cc. of water on the steam bath for 102 hours. The hydrolysate, which gave no biuret test, was treated as described above, except that alcohol was added to depress the solubility of the cystine instead of acetone. After standing in the ice box for 14 days and at room temperature for 10 days, a precipitate, which weighed 3.237 gm. after drying, was filtered off and carefully washed. This precipitate was dissolved in dilute hydrochloric acid. Colorimetric determination showed the presence of 2.78 gm. of cystine, corresponding to 11.6 per cent of the hair, or about 68 per cent of the amount of cystine found directly by the regular Folin-Looney procedure. The sulfur analysis of the precipitate corresponded to 2.75 gm. of cystine, a value in good agreement with the colorimetric determination.

The filtrate, after the removal of the impure cystine, was analyzed as in the first experiment, and contained 867 mg. of cystine by the colorimetric method. This gave a total cystine content of the hair of 3.62 gm., or 15.1 per cent of the original sample, a considerable reduction of the value found by direct analysis (17.0 per cent). This difference is to be explained in part, at least, by the fact that a fairly strong test for hydrogen sulfide was given by the vapors above the filtrate after the alcohol had been distilled, prior to the colorimetric determination. The solution did not contain sulfides or mercapto compounds, however, as there was a negligible amount of color developed with the uric acid reagent in the absence of the sulfite.

The sulfur content of the filtrate was 304 mg., which corresponds to 810 mg. of cystine. A summation of all the sulfur values gives a total of 1.039 gm. of sulfur, or 4.33 per cent of the original sample. The value originally found was 5.05 per cent.

We believe that the correspondence between the cystine contents of the filtrates as determined colorimetrically and as cal-

culated from the analyses of the sulfur content is too close to be accidental. In our opinion, these experiments indicate that the precipitation of cystine is incomplete and that a considerable proportion of the cystine remains in the filtrate. While we cannot of course, be certain that cystine is the only substance in the filtrate which reacts with the Folin-Looney reagent in the presence of sulfite, it seems hardly possible that so close a correspondence between the cystine content of the filtrate as determined by the two methods could exist, if any considerable amounts of sulfur compounds other than cystine were present. Further work on the application of the colorimetric method of analysis of the cystine content of proteins is in progress.

SUMMARY.

1. The cystine content of various types of keratins has been determined colorimetrically by the method of Fohn and Looney. Human hair gave values from 15.6 to 21.2 per cent; sheep wool, 8.0 to 10.9 per cent; feathers, 7.05 to 12.2 per cent; rabbit hair, 11.9 to 14.0 per cent, tortoise shell, 6.4 to 8.1 per cent; rat hair, 14.1 per cent, cat hair, 13.1 per cent, dog hair, 19.0 per cent.

2. Although the sulfur and nitrogen values of keratins from individuals of a species varied rather widely, the ratio of nitrogen to sulfur was relatively constant for that keratin.

3. Three samples of human skin, and one of human connective tissue, were analyzed. The cystine content was lower (1.82 to 2.34 per cent) and the nitrogen to sulfur ratio (23 to 25) higher than those of human hair.

4. No relationship was evident between the cystine content of human hair and the color of the hair, age, or sex of the individual.

5. Little evidence was obtained of the existence of significant amounts of non-cystine sulfur in the keratins studied.

6. The cystine contents of two samples of hair were determined by the older method of precipitation of the impure cystine and gravimetric determination of the sulfur content of the precipitate. It was found that the difference between the colorimetric value and the value calculated from the sulfur content of the precipitate could be accounted for almost completely by the amount of cystine (and sulfur) remaining in the filtrate after the removal of the precipitate.

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THE FATE OF SUGAR IN THE ANIMAL BODY.

VI. SUGAR OXIDATION AND GLYCOGEN FORMATION IN NORMAL AND INSULINIZED RATS DURING THE ABSORPTION OF FRUCTOSE.

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The fate of ingested glucose has been reported on a former occasion (1). It was found that sugar oxidation plus glycogen formation accounted for 90 per cent of the glucose absorbed during 4 hours. When insulin was injected there was a marked increase in glucose oxidation with a corresponding decrease in glycogen formation. The chief organ in which glycogen failed to be deposited during insulin action was the liver, while the increased sugar oxidation occurred in the muscles. There was a shift in the disposal of glucose from the liver into the muscles as the result of a surplus of insulin. Other experiments (2) made it probable that a similar shift occurred in the case of fructose. During 4 hours of fructose absorption the insulinized rats deposited 108 mg. less of liver glycogen than the normal rats. It was assumed that the fructose that failed to be retained as liver glycogen was disposed of by increased oxidation. In order to test this assumption, recovery experiments with fructose on normal and insulinized rats were undertaken.

EXPERIMENTAL.

The technique described in the second paper of this series (1) has been followed exactly. Male rats of 110 to 140 gm. of body weight, fasted previously for 48 or 24 hours, were used. The urine from the 21st to the 45th (or from the 4th to the 21st) hour of fasting was collected quantitatively for nitrogen determinations. In the case of the 48 hour fasting rats the urine was also analyzed

for total acetone bodies by means of the method of Van Slyke (3). A fore period of 2 to 3 hours was then made in a Haldane type of metabolism apparatus, followed immediately by the sugar feeding. The respiratory exchange during the 4 hours of fructose absorption was determined in a closed type of metabolism apparatus, a description of which has been given in a former paper (1). In this metabolism apparatus the oxygen consumption is determined by two independent methods. A check between the two methods insures that a leak or an incomplete absorption of CO_2 did not occur. The average difference between the two determinations for the twenty metabolism experiments recorded in this paper was

TABLE I
Glycogen Content of Male Rats, Fasted Previously for 48 or 24 Hours.

Glycogen in per cent of body weight	
48 hr fasting	24 hr fasting
0 131	0 140
0 146	0 161
0 160	0 152
0 118	0 147
0 148	0 134
0 133	0 159
0 119	0 158
Average 0 136 \pm 0 013 gm	0 150 \pm 0 008 gm.
Average of former exper- iments (1) 0 114 \pm 0 011 "	

1.16 per cent. After the completion of the 4 hour metabolism period the animal was killed, the intestinal tract removed for the determination of the amount of sugar absorbed, and the rest of the body worked up for glycogen. Control rats, fasted previously for 48 or 24 hours respectively, were used for the determination of the preformed glycogen (Table I). The total amount of glycogen found at the end of the 4 hour sugar absorption period was corrected for the preformed glycogen.

The amount of sugar oxidized was calculated from the non-protein respiratory quotient, using 8.488 mg of O_2 and 9.348 mg. of CO_2 as the equivalent of 1 mg. of urine N. The following con-

version factors have been used: 1 liter of O_2 = 1.429¹ gm.; 1 liter of CO_2 = 1.9768¹ gm.

Rats fasted for only 24 hours have previously not been used for sugar recovery experiments. It will be noted in Table I that the variations in the preformed glycogen of rats fasted for 24 hours is small (only ± 8 mg. of glycogen). The respiratory quotients of the fore period indicate that rats after a 24 hour fast oxidize a negligible amount of carbohydrates. These two facts made it possible to use rats fasted for only 24 hours, with the added advantage that there was no danger from ketosis. The changes in the carbohydrate metabolism of 48 hour fasting rats suffering from ketosis have been described in a previous paper (4). In the winter months about 10 per cent of the 48 hour fasting rats develop ketosis in the last 24 hours of fasting. In summer the number of rats developing ketosis reaches 90 per cent. All the experiments reported in this paper were made in winter. Rats that excreted more than 4 mg. of total acetone bodies per 100 gm. of body weight per 24 hours, which coincides with a respiratory quotient below 0.700, were not used for recovery experiments.

Recovery Experiments on Rats Fasted Previously for 48 Hours.

The first point of interest is that rats fasted for 48 hours absorb decidedly less fructose than rats fasted for 24 hours. In the former case the average amount of fructose absorbed per 100 gm. of body weight per hour is 101 mg., while in the latter case the value is 148 mg. The meaning of this difference gains more significance on considering that 48 hour fasting rats use less oxygen than 24 hour fasting rats (180 mg. per 100 gm. per hour against 220 mg. per 100 gm. per hour). Fasting, which reduces more or less the functional activity of all body cells, has an especially profound influence on the rate of intestinal absorption. In keeping with this general effect of fasting is the result that rats fasted for 48 hours oxidize less fructose than rats fasted for 24 hours.

¹ These are the figures given in Landolt-Bornstein (Landolt, H., Bornstein, R., Roth, W. A., and Scheel, K., *Physikalisch-chemische Tabellen*, Berlin, 5th edition, 1923) and in the Smithsonian physical tables (Fowle, F. E., 1916, lxiii, No. 2269, 6th edition, Washington). Landolt-Bornstein (3rd edition, 1905) and Lusk (*The elements of the science of nutrition*, Philadelphia and London, 3rd edition, 1923) give 1 liter of CO_2 = 1.965 gm.

Tables II and III summarize the experiments on 48 hour fasting rats. The animals without insulin absorbed in 4 hours an average of 410 mg, oxidized 42 mg, and converted into glycogen 201 mg. of fructose, respectively. This corresponds to a recovery of the absorbed fructose of 59.2 per cent or to a loss of fructose of 167 mg. The insulinized rats absorbed 403 mg, oxidized 231 mg, and converted into glycogen 122 mg of fructose, a recovery of the absorbed fructose of 87.8 per cent or a loss of 50 mg of fructose. The mechanism by which part of the absorbed fructose disappears in the non-insulinized animals without being either excreted, oxidized, or converted into glycogen, is very mysterious.

TABLE II

Recovery Experiments with Fructose on 48 Hour Fasting Rats.

All values are per 100 gm of body weight per 4 hours

Fore period		Fructose absorption period								
O ₂	R Q	Fructose absorbed	Fructose oxidized	Glycogen formed	Fructose recovered		O ₂ *	R Q	Urine N	Blood sugar
gm		gm	gm	gm	gm	per cent	gm		mg	mg
0 725	0 702	0 417	0 058	0 223	0 281	67 2	0 814	0 740	11 04	137
0 783	0 707	0 394	0 060	0 158	0 218	55 4	0 896	0 739	11 56	146
		0 426	0 016	0 236	0 252	59 1	0 879	0 724	12 80	154
0 695	0 708	0 404	0 036	0 187	0 223	55 1	0 748	0 735	12 88	161
0 734	0 706	0 410	0 042	0 201	0 243	59 2	0 834	0 734	12 07	149

* The average values of the direct and indirect O₂ determinations are recorded. The average difference between the two determinations of this series was 1.42 per cent. Total acetone bodies per 100 gm of body weight per 24 hours were 2.6, 1.8, 2.4, and 0.4 mg, respectively.

Whatever the final explanation, the fact remains that insulin injections, by increasing the amount of sugar oxidized, lead to a satisfactory recovery of the absorbed sugar. The same phenomenon has been observed previously with glucose on rats suffering from ketosis (4). Without insulin only 70.9 per cent of the absorbed glucose could be recovered, while with insulin the recovery reached 90 per cent. Ketosis, in the sense as it was defined in the paper just cited, was not present in the experiments here reported. The respiratory quotients of the fore period were above 0.700 and the excretion of total acetone bodies in the 24 hours preceding the experiments did not exceed 4 mg.

In order to determine what happened to the fructose lost in the experiments without insulin, the same amount of fructose (*i.e.* 400 mg. per 100 gm. of body weight) was fed that was absorbed in 4 hours, but the respiratory exchange determined for 6 hours. In all other experiments the animals were killed while active absorption was still going on; that is, when there was still a surplus of sugar in the intestine. In this case the absorption came to an end 2 hours before the metabolism experiment was interrupted. In other words, during the last 2 hours of the 6 hour metabolism pe-

TABLE III

Recovery Experiments with Fructose Plus Insulin on 48 Hour Fasting Rats.

8 units of insulin per 100 gm. of body weight were injected simultaneously with the sugar feeding. All values are per 100 gm. of body weight per 4 hours

Fore period		Fructose absorption period								
O ₂	R Q	Fructose absorbed	Fructose oxidized	Glycogen formed	Fructose recovered		O ₂ *	R Q	Urine N	Blood sugar
gm		gm	gm	gm	gm	per cent	gm		mg	mg
0 697	0 703	0 421	0 279	0 096	0 375	89 1	0 814	0 824	10 64	78
0 730	0 723	0 429	0 216	0 174	0 390	90 9	0 776	0 805	11 12	59
0 718	0 703	0 435	0 227	0 110	0 337	77 4	0 809	0 805	11 12	83
0 674	0 714	0 365	0 258	0 092	0 350	96 0	0 726	0 829	10 80	60
0 695	0 708	0 364	0 174	0 138	0 312	85 7	0 759	0 791	11 84	77
0 703	0 710	0 403	0 231	0 122	0 353	87 8	0 777	0 811	11 10	71

* The average values of the direct and indirect O₂ determinations are recorded. The average difference between the two determinations was 1.56 per cent. Total acetone bodies per 100 gm. of body weight per 24 hours were 1.7, 2.3, 2.4, 2.3, and 2.8 mg, respectively.

riod there was no absorption of fructose taking place. The purpose of these experiments was to see whether the fructose was actually lost due to some unknown metabolic process or whether there was merely a discrepancy between the rate at which fructose was absorbed and the rate at which the sugar was disposed of in the tissues. By extending the metabolism period beyond the period of active absorption a chance was given for any lag in the disposal of sugar to disappear. Conceivably, a lag might have occurred either in the sugar oxidation or in the glycogen formation.

The results per 100 gm. of body weight per 6 hours, as an average of six experiments, were as follows: fructose absorbed, oxidized, and converted into glycogen, 405, 146, and 239 mg., respectively; fructose recovered, 95.0 per cent; fructose lost, 20 mg.; blood sugar, 130 mg. per cent.

It will be noted that in these experiments 146 mg. of fructose were oxidized as compared with 42 mg. of fructose in the 4 hour experiments without insulin. Due to this increase in sugar oxidation 95.0 per cent instead of only 59.2 per cent of the absorbed sugar was recovered. A loss of only 20 mg. of fructose occurred as compared with a loss of 167 mg. of fructose in the 4 hour experiments without insulin. The difference of 147 mg. appears for the most part on the side of oxidation, since the glycogen formation was within 38 mg. the same. By giving the organism a chance to catch up with the absorbed sugar an additional amount of 104 mg. of fructose is oxidized. The original loss of fructose was, therefore, not due to some unknown metabolic process but to a lag in sugar oxidation. The question is in which form the sugar is retained. The blood sugar values observed in these experiments, in so far as they reflect the sugar concentration in the tissue, make it improbable that fructose was retained in the tissues as such. One is led to believe that sugar must enter into some form of combination (possibly with phosphoric acid) before it can undergo oxidation. The guess is that the lag in fructose oxidation is due to the retention of part of the fructose in a combined form, representing the first step in the transformation of sugar on its path to oxidation. If enough time is allowed to elapse or if insulin is injected the fructose combination is broken up and the sugar appears as oxidized. It is interesting to note that in the 4 hour experiments with insulin more fructose was oxidized than in the experiments with the 4 hour absorption and 6 hour metabolism period. A surplus of insulin increases the rate of fructose oxidation to a marked extent. One may infer from this that the lag in fructose oxidation is due to an insufficient supply of insulin brought about by a longer period of fasting. As will be shown later, a lag in fructose oxidation does not occur in rats fasted for only 24 hours. It would be idle at this stage to speculate on the mechanism of insulin action. The suggestion here given may merely serve as a basis for further investigation.

Possibly a method has been found, whereby sugar oxidation can be caught in its first stage.

The lag of fructose oxidation in 48 hour fasting rats without insulin cannot always be reproduced to the same extent. After completion of the above experiments, a new series of 48 hour fasting rats was begun. These animals showed a higher oxygen consumption in the fore period (204 mg. per 100 gm. per hour against 180 mg. in the former series) and absorbed fructose at a greater rate. Possibly this was due to the fact that the animals were of a younger age. The average values of five experiments per 100 gm. of body weight per 4 hours were as follows: fructose absorbed, oxidized, and converted into glycogen, 482, 129, and

TABLE IV

Amount of Glycogen Formed in Liver and in Rest of Body in 48 Hour Fasting Rats during 4 Hours of Fructose Absorption.

	Liver in per cent of body weight	Glycogen in liver	Glycogen in other tissues	Total glycogen	Liver in per cent of total glycogen	Other tissues in per cent of total glycogen.
		gm	gm	gm		
Fructose alone	3 23	0 127	0 074	0 201	63 4	36 6
“ plus insulin	2 82	0 019	0 103	0 122	15 6	84 4
Difference		-0 108	+0 029	-0 079		

259 mg., respectively; fructose recovered, 80.5 per cent; fructose lost, 94 mg; blood sugar, 147 mg. per cent.

The individual experiments were consistent with each other. In all cases the recovery of the absorbed fructose was better than in the first series of experiments without insulin. This was mainly due to an increase in fructose oxidation. It should be noted, however, that the fructose oxidation did not attain the same rate as in the insulinized animals of the first series. The latter oxidized 102 mg. more fructose in spite of the fact that they absorbed less fructose.

The relative amounts of glycogen deposited in the liver and in the rest of the body tissues during 4 hours of fructose absorption are shown in Table IV. The values for the total glycogen are those of Tables II and III, while the values for the liver glycogen

have been taken from a former series of experiments (2). The fact that stands out clearly is that the difference in the total glycogen of the normal and insulinized rats is entirely accounted for by the difference in liver glycogen

Recovery Experiments on Rats Fasted Previously for 24 Hours.

The individual experiments with 24 hour fasting rats are given in Tables V and VI, the average values in Table VII. Fig 1 is a

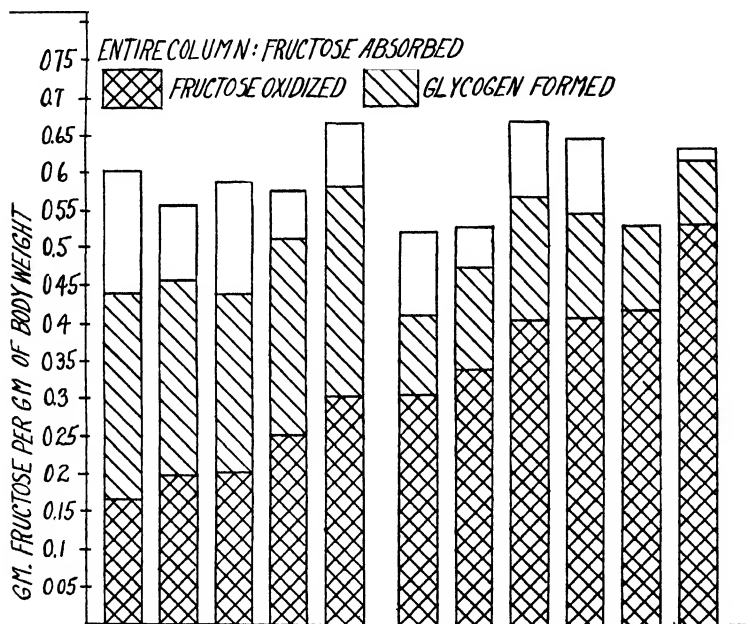


FIG 1 Graphic illustration of the experiments in Tables V and VI. Bars 1 to 5 are the experiments without and Bars 6 to 11 the experiments with insulin

bar diagram illustrating these experiments. The average amount of fructose absorbed was 598 mg for the normal and 585 mg. for the insulinized rats. Due to the shorter period of fasting there was no appreciable lag in fructose oxidation. In the normal series 81.2 per cent of the absorbed fructose was recovered against 89.7 per

cent in the insulinized series. Taking the equal absorption and nearly equal recovery as basis, any difference between normal and insulinized rats must show itself in the relative amounts of fructose oxidized and glycogen formed. The insulinized animals oxidized, as an average, 177 mg. more fructose and deposited 138 mg. less glycogen than the normal animals. By dividing glycogen formed fructose oxidized one learns how many mols of fructose are converted into glycogen per mol of fructose oxidized. This was 1.16

TABLE V
Recovery Experiments with Fructose on 24 Hour Fasting Rats
All values are per 100 gm. of body weight per 4 hours

Fore period		Fructose absorption period										
O ₂	R Q	Fructose absorbed	Fructose oxidized	Glycogen formed	Fructose recovered		Glycogen formed Fructose oxidized	O ₂ *	R Q	Urine N	Blood sugar	
gm		gm	gm	gm	gm	per cent	gm	gm		mg	mg	
0 921	0 701	0 603	0 165	0 273	0 438	72 5	1 65	0 925	0 775	15 24	148	
0 797	0 702	0 557	0 198	0 258	0 456	81 7	1 30	0 788	0 801	12 08	149	
0 899	0 724	0 587	0 201	0 236	0 437	74 6	1 17	0 872	0 792	15 12	142	
0 915	0 707	0 575	0 253	0 258	0 511	89 0	1 02	0 841	0 813	13 20	137	
0 798	0 721	0 666	0 302	0 280	0 582	87 3	0 93	0 835	0 832	13 12	150	

* Average value of the direct and indirect O₂ determination. The average difference between the two determinations was 1.1 per cent. The difference in the R Q, when calculated from the average direct and indirect O₂ values, was 0 009.

mols for the normal and 0.3 mols for the insulinized animals. On considering the results obtained in a former paper (1) with glucose one may conclude that the effect of a surplus of insulin on the disposal of glucose and fructose in the animal body is the same. In both cases insulin inhibits glycogen formation in the liver and leads to an increased oxidation of sugar in the muscles.

Three experiments (one without and two with insulin) belonging to the 24 hour series had to be ruled out. In the former case the blood sugar was as high as 244 mg. Apparently fructose as such

had been retained in the tissues, since the recovery was only 69 per cent. In one of the insulinized rats the amount of fructose absorbed departed too greatly from the average value of the series (776 mg were absorbed against the average of 585 mg.). The second insulinized rat oxidized for some unknown reason only 130 mg of fructose against the average of 224 mg. for the normal and 401 mg for the insulinized rats.

TABLE VI

Recovery Experiments with Fructose Plus Insulin on 24 Hour Fasting Rats.

8 units of insulin per 100 gm of body weight were injected simultaneously with the sugar feeding All values are per 100 gm of body weight per 4 hours

Fore period		Fructose absorption period									
O ₂	R Q	Fructose absorbed	Fructose oxidized	Glycogen formed	Fructose recovered		Glycogen formed Fructose oxidized	O ₂ *	R Q	Urine N	Blood sugar
gm		gm	gm	gm	gm	per cent	gm	gm		mg	mg
0 771	0 709	0 520	0 305	0 105	0 410	78 9	0 34	0 829	0 833	12 48	66
0 875	0 702	0 526	0 338	0 133	0 471	89 6	0 39	0 929	0 832	13 60	71
1 086	0 729	0 666	0 403	0 162	0 565	84 8	0 41	0 936	0 857	15 44	85
0 956	0 701	0 643	0 408	0 135	0 543	84 6	0 33	0 945	0 853	14 24	89
0 831	0 709	0 528	0 416	0 122	0 538	102 2	0 29	0 811	0 882	16 06	67
0 845	0 719	0 629	0 531	0 081	0 612	98 9	0 15	0 929	0 897	14 56	68

* Average of direct and indirect O₂ determination The average difference between the two determinations was 0 71 per cent The difference in the R Q, when calculated from the average direct and indirect O₂ values, was 0 006

SUMMARY AND CONCLUSIONS

1. Rats fasted previously for 48 hours use less oxygen, and, after sugar feeding, absorb less fructose and oxidize less fructose than 24 hour fasting rats.

2. In 48 hour fasting rats part of the absorbed fructose disappears without being either excreted, oxidized, or converted into glycogen Sugar oxidation plus glycogen formation accounted for only 59.2 per cent of the fructose absorbed during 4 hours. When

insulin was injected the recovery of the absorbed sugar reached 87.8 per cent. This was due to an increase in fructose oxidation.

3. When 48 hour fasting rats were fed just enough fructose to

TABLE VII

Average of Experiments in Tables V and VI.

All values are per 100 gm of body weight per 4 hours.

	Fructose alone (5 rats)	Fructose plus insulin (6 rats)
Fore period:		
O ₂	0 866 gm.	0 894 gm.
CO ₂	0 852 "	0 880 "
R Q	0 711	0 711
Total calories	2 96	3 06
Fructose absorption period:		
Fructose absorbed	0 598 gm.	0 585 gm.
" oxidized	0 224 "	0 401 "
Glycogen formed	0 261 "	0 123 "
Fructose recovered	0 485 "	0 524 "
	(81.2 per cent).	(89.7 per cent).
" lost	0 113 gm.	0 061 gm.
Glycogen formed		
Fructose oxidized	1 16	0 30
Blood sugar.		
O ₂	0 145 gm.	0 074 gm.
CO ₂	0 852 "	0 896 "
CO ₂ . . .	0 945 "	1 066 "
R Q	0 802	0 859
Urine N	13 75 mg.	14 39 mg.
Non-protein O ₂ . . .	0 735 gm.	0 774 gm.
" CO ₂	0 816 "	0 931 "
" R Q	0 803	0 870
Protein oxidized	0 084 gm	0 088 gm.
Fat oxidized	0 173 "	0 123 "
Calories from protein*	0 34	0 36
" " fat†	1 63	1 16
" " fructose‡. . . .	0 84	1 50
Total calories	2 81	3 02

* Heat from protein (urine N \times 24.98 calories).

† Heat values for animal fat 9.4 calories.

‡ Heat value for fructose 3.74 calories.

let the absorption proceed for 4 hours but the respiratory exchange was determined for 6 hours, 95 per cent of the absorbed sugar was accounted for. Absorption and glycogen formation being nearly the same as in the 4 hour experiments, the better recovery was due

to the fact that a larger amount of fructose was oxidized. By extending the measurement of the metabolism beyond the period of active absorption the organism was enabled to catch up with the sugar that had been absorbed. Therefore, the low recovery in the 4 hour experiments without insulin was the result of a lag in fructose oxidation and was not due to some unknown metabolic process

4. In the 24 hour fasting rats fructose oxidation plus glycogen formation accounted for 81.2 per cent of the absorbed sugar in the normal and for 89.7 per cent in the insulinized rats. For the equal amount of fructose that was absorbed during 4 hours, the insulinized rats oxidized more fructose and deposited correspondingly less glycogen than the normal rats. The quotient $\frac{\text{glycogen formed}}{\text{fructose oxidized}}$ was 1.16 for the normal and 0.3 for the insulinized rats.

5. It is concluded that a surplus of insulin leads to an increased oxidation of fructose

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A STUDY OF HUMAN RED BLOOD CELL PERMEABILITY.

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In their recent studies (20) on the water and electrolyte distribution between cells and serum, Van Slyke, Wu, and McLean assumed for their theoretical considerations that the red blood cells were impermeable to the cations sodium and potassium. They showed that when the serum *anion* concentration was changed in whole blood CO₂ saturation experiments, the reestablishment of electrolyte equilibrium could be entirely accounted for by the transfer of water and anions across the cell membrane. Several previous investigators (5, 6, 11, 12, 18) had reported similar findings in CO₂ saturation experiments, but Hamburger (8, 9) found that when the concentration of the *cations* in the serum was changed by adding sodium or potassium salts or water to whole blood, both basic and acid ions appeared to traverse the cell membrane in the electrolyte readjustment. At present, therefore, since Hamburger's latter findings stand undisputed, any assumption of the impermeability of the red blood cells to sodium or potassium seems open to doubt.

Since this question is of fundamental importance to an understanding of electrolyte equilibrium, the present writers have repeated some of Hamburger's experiments using the more refined laboratory procedures that have been recently acquired, because it was believed on theoretical grounds that the red blood cells were impermeable to cations within the limits of electrolyte change found in human disease. They found that when the electrolyte equilibrium was disturbed by the addition of salt or water to blood even beyond the extreme limits of variation recorded in human blood, the red blood cell membrane apparently remained impermeable to the cations sodium and potassium.

It seemed of unusual interest to include in the experiments the salts of both sodium and potassium inasmuch as the extraordinary and little understood separation of potassium within the cells and sodium in the serum and tissue fluids of the body suggested that the cell membrane might show a selective permeability for potassium.

EXPERIMENTAL.

Method.

Human venous blood was collected into large Pyrex tubes and defibrinated with a glass stirring rod. From 70 to 120 cc. of blood were taken for each experiment depending upon whether the blood was divided into two or three samples. In either case, one sample was analyzed directly. In three experiments equimolar amounts of similar sodium and potassium salts were added to a second and a third sample. In the remaining experiments, a single salt or water was added to the second sample. Salts of chloride and carbonate were used because of the ease of determining their anion distribution between the cells and serum. The amounts of salt added varied from 21.9 to 100.4 milli-equivalents of base in 1000 cc. of serum. The smaller amounts of carbonate salt added represent the maximal amount of CO_2 that could be conveniently analyzed. Water was added in the proportion of 250 cc. of H_2O per 1000 cc. of blood. The dried weighed salt and the measured amount of water were added directly to the whole blood without causing noticeable hemolysis by first centrifuging down the cells and adding the salts or water to the supernatant serum, and then remixing the cells and serum. In order to prevent any organic change within the blood system during the delays between the various determinations, the blood was kept on ice until ready to use and then quickly brought to room temperature.

All specimens were saturated with 40 mm. of CO_2 in air at 38°C . by the technique previously described (1). Oxygen capacity determinations were done to assure an equal hemoglobin concentration in the duplicate or triplicate specimens. Cell volume and serum protein were determined to indicate the water shift between the cells and serum. CO_2 content and chloride concentration were determined in the whole blood and the serum to show the change

in their distribution between the cells and serum. The total base in the serum was determined to indicate whether there had been any exchange of base across the cell membrane.' And finally, the sodium concentration of the serum was determined in five experiments to show whether in case the serum total base content remained constant there occurred an equimolar shift of sodium and potassium in opposite directions across the membrane.

Analytical Procedures.

Oxygen capacity was determined in the Van Slyke constant pressure pipette by a method devised by Lundsgaard and Neill¹ Blood cells from which the plasma had been removed were used for the O₂ determination as described in a previous communication (15). Where whole blood and diluted cells were compared, the agreement was fairly satisfactory

Cell volume was determined by using a Daland hematocrit fitted to a No. 1 International Equipment Company centrifuge Duplicate determinations were carried out on each blood sample and always agreed within 1 volume per cent

*Serum proteins*² were determined by macro-Kjeldahl procedure using about $\frac{1}{2}$ cc samples of serum diluted with physiological saline

CO₂ was determined by the Van Slyke method (22) in a calibrated constant volume pipette

*Chlorides*³ were determined by the Van Slyke method (21) A few of the whole blood chlorides were determined by the Whitehorn method (23)

Total base was determined by a modification of Stadie and Ross's adaptation of Fiske's urine method (16)

Sodium was determined by the following modification of the Kramer and Gittleman method (14)

Solutions—The alcohol, KOH, and Na₂S₂O₃ solutions were prepared exactly as described by Kramer and Gittleman (14) 2 per cent KI was used in place of 20 per cent Great difficulty was experienced

¹ Personal communication.

² The serum protein results in Experiments 1, 2, and 3 are probably too low because no superoxol was added to assure complete digestion In the remaining experiments, where superoxol was used, the results are believed to be more accurate

³ The whole blood chlorides in the first three experiments were determined by Whitehorn's method It was discovered that added NaCl was not always recovered completely by this method Hence the Van Slyke method was adopted for the last five experiments for both whole blood and serum Inasmuch as the serum chlorides in the first three experiments were determined by the Van Slyke method which gave good checks, the cell chlorides in these experiments were recalculated on the basis of the known serum volume, serum chloride, and added chloride

in obtaining a satisfactory potassium pyroantimonate solution. The Baker salt, suggested by Kramer and Gittleman, gave good results with only one of three lots. When the material was analyzed for its antimony content, varying amounts of Sb were found. The use of the Baker salt was therefore replaced by that of a Kahlbaum salt, pure $K_2H_2Sb_2O_7$. Made up as described by Kramer and Gittleman, this solution gave satisfactory results. Starch was found to be unnecessary in the final titration.

Analysis—2.5 cc of serum were ashed as described by Kramer (13). Kramer and Gittleman (14) determined sodium directly on the unashed serum. This method, however, gives lower results than when the serum is ashed, and recovery of added sodium is not quantitative. Therefore, the present writers believe, as was pointed out by Balint (2), that more complete sodium recovery is obtained by ashing the serum. The ash is dissolved in 2.5 cc of 0.1 N HCl. 1 cc portions are placed in 25 cc conical Pyrex centrifuge tubes. (These tubes may not be used for other analyses and must be washed with water and brush only.) 2 drops of 10 per cent KOH (Na-free) are added and the contents of the tube thoroughly mixed by stirring with a glass rod. 5 cc of $K_2H_2Sb_2O_7$ solution are added. 1.5 cc of 95 per cent C_2H_5OH are added with constant stirring. The tubes are allowed to stand for 30 minutes and are then centrifuged for 5 minutes. The supernatant fluid is decanted and the tube is drained on filter paper. The precipitate is washed with 5 cc of 30 per cent C_2H_5OH and the tube is again centrifuged, the fluid decanted, and drained as before. The precipitate is dissolved in 2.5 cc of 10 N HCl and 2.5 cc of H_2O . 2 cc of 2 per cent KI are added and the solution is titrated immediately with 0.1 N $Na_2S_2O_3$ until the iodine color disappears.

The above proportions of reagents will adequately take care of 2.00 to 3.75 mg of Na. If the presence of less Na is suspected more serum must be used. If 1 cc of serum contains more than 3.75 mg of Na, the ash should be dissolved in 5 cc of 0.1 N HCl and 2 cc portions (equivalent to 1 cc of serum) used in the analysis. In this case double quantities of all the reagents must be used.

Using 1 cc of HCl-containing ash and the amounts of reagents described above, the blank is 6 mg of Na per 100 cc of serum. Using 2 cc of HCl-containing ash and double the reagents, the blank is 12 mg. The method yields, as Balint (2) showed, 103 per cent sodium.

Calculation—When the equivalent of 1 cc of serum is used

$$\frac{(\text{Cc } Na_2S_2O_3 \times 115) - 6}{1.03} = \text{mg Na per 100 cc serum}$$

Results.

The results of eight experiments are reported, three in which equimolar amounts of similar sodium and potassium salts were added to like blood samples, five in which a single salt or water was added and sodium was determined.

TABLE I.

Complete Electrolyte Determinations Expressed in Various Units Conventionally Used.

M.-eq. = milli-equivalent, the unit for all monovalent ions, represents one-thousandth of the gram-molecular weight. The numbers following the amount of Na_2CO_3 and K_2CO_3 added, represent m.-eq. of sodium or potassium. Total base = the base-combining power of the serum expressed as m.-eq per liter.

Experiment No	Salt or water added to blood		Cell volume		Oxygen capacity		Carbon dioxide		Chloride		Protein serum	Na serum	Total base serum
							Blood	Serum	Blood	Serum			
	m.-eq or cc per l		vols per cent	vols per cent	vols per cent	vols per cent	vols per cent	vols per cent	gm per l	gm per l	per cent	m.-eq per l	m.-eq per l
1			45 35	20 00	50 7	58 6	2 61	3 61	6 72				154 2
	NaCl	34 7	38 73	20 06	49 4	59 5	3 66	5 04	6 26				195 5
	KCl	34 7	38 85	19 94	50 0	59 6	3 63	5 04	6 37				192 0
2			57 80	24 06	48 3	61 4	2 47	3 50	7 08				155 0
	Na_2CO_3	9 2	54 55	23 82	61 7	78 0	2 47	3 60	6 76				164 0
	K_2CO_3	9 2	54 25	23 88	61 5	78 4	2 47	3 59	6 11				165 5
3			58 85	23 80	48 8	61 5	2 52	3 52	8 02				150 4
	Na_2CO_3	13 9	53 40	24 00		86 9	2 52	3 61	6 79				161 6
	K_2CO_3	13 9	54 20	24 10	67 8	86 8	2 52	3 61	7 30				161 0
4			38 45	16 50	51 1	60 3	2 76	3 66	6 74	146 5			158 8
	NaCl	50 8	34 50	16 59	52 3	61 0	3 88	4 92	6 32	181 7			193 2
5			37 2	15 54	54 0	63 4	2 87	3 56	6 13	145 2			158 2
	KCl	51 2	32 3	15 56	54 4	63 2	4 02	4 94	5 75	133 0			193 0
6			45 3	20 27	47 1	55 8	2 76	3 68	6 19	140 0			155 2
	Na_2CO_3	23 6	42 6	20 39	65 0	80 5		3 72	5 85	155 6			171 6
7*			42 2	18 06	45 8	53 6	2 71	3 63	6 56	149 6			149 4
	H_2O	250	40 5	13 96	38 1	45 2	2 21	2 87	5 10	115 4			116 8
8			43 8	18 98	42 2	50 6	2 75	3 68	7 10	141 7			153 6
	H_2O	250	41 6	15 20	36 7	41 8	2 29	2 85	5 55	109 3			119 0

* In Experiment 7, due to a technical error in the delivery of the blood from sampling bulb before it was properly mixed, the hematocrit and whole blood CO_2 and Cl were inaccurate. These determinations were, therefore, corrected on the basis of the serum protein change and the serum CO_2 and Cl.

TABLE II

Concentration of Bicarbonate, Chloride, Sodium, and Total Base in Cells and Serum before and after Addition of Salt.

The sodium and total base "calculated" represent their expected concentrations in the serum if there is no transfer of base across the cell membrane.

Experiment No	Salt added to blood		Serum volume	HCO ₃		Cl		Na serum		Total base serum	
				Cells	Serum	Cells	Serum	Found	Calculated	Found	Calculated
	<i>m -eq per l</i>		<i>per cent</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>	<i>m -eq per l</i>
1			54 65	17 3	24 9	39 5	101 8			154 2	
	NaCl	34 7	61 27	13 9	25 3	40 7	142 0			195 5	193 5
	KCl	34 7	61 15	14 5	25 3	40 7	142 3			192 0	194 0
2			42 20	16 2	26 2	49 3	99 0			155 0	
	Na ₂ CO ₃	9 2	45 45	20 0	33 2	43 6	101 4			164 0	167 5
	K ₂ CO ₃	9 2	45 75	20 0	33 2	43 6	101 3			165 5	167 5
3			41 15	16 8	26 2	50 8	99 4			150 4	
	Na ₂ CO ₃	13 9	46 60	21 8	37 5	44 5	102 0			161 6	164 0
	K ₂ CO ₃	13 9	46 80	21 8	37 5	44 5	102 0			161 0	164 0
4			61 55	15 2	25 6	37 3	103 1	146 5		158 8	
	NaCl	50 8	65 50	15 0	26 0	53 6	138 7	182 5	185 5	193 2	196 0
5			62 80	16 0	26 8	47 9	100 4	145 2		158 2	
	KCl	51 2	67 70	14 9	26 8	59 4	139 2	133 0	134 7	193 2	193 8
6			54 70	15 2	23 6	46 6	103 7	140 0		155 2	
	Na ₂ CO ₃	23 6	57 40	18 6	34 6	41 1	105 0	155 6	156 0	171 6	169 8

Sodium and Total Base Calculated.

$$B_2 = \frac{B_1 V_1 + B_a}{V_2}$$

Where B_1 = Sodium or total base found in 1000 cc of untreated serum.

B_a = M -eq base added per liter

B_2 = Sodium or total base calculated in 1000 cc. of treated serum.

V_1 = Serum volume per cent in untreated blood.

V_2 = " " " " " treated blood.

Table I contains the complete electrolyte determinations.

Tables II and III represent changes in serum volume, cell and serum bicarbonate and chloride concentration, and sodium and total base concentration produced by the addition of salt or water.

TABLE III

Concentration of Bicarbonate, Chloride, Sodium, and Total Base in Cells and Serum before and after Addition of Water.

The cell volume is expressed in relation to 125 cc of blood in the specimens to which water has been added, instead of 100 cc. as it is in the untreated specimens. The figures in parentheses represent the concentration of HCO_3 and Cl calculated on the assumption that there is no transfer across the cell membrane.

Experiment No	Water added to blood	Cell volume	HCO_3		Cl		Na serum		Total base serum	
			Cells	Serum	Cells	Serum	Found	Calculated	Found	Calculated
			m-eq per l	m-eq per l	m-eq per l	m-eq per l	m-eq per l	m-eq per l	m-eq per l	m-eq per l
7	cc per l	per cent	42 20	14 6	22 6	38 5	102 4	149 6	149 4	
	H ₂ O 250	50 60	11 3	18 9 (17 6)	34 6	80 9 (79 6)	115 4	116 2	116 8	115 8
8		43 83	13 0	21 3	43 6	103 6	141 7		153 6	
	H ₂ O 250	52 00	12 1	17 4 (16 4)	41 9	80 4 (79 8)	109 3	109 0	119 0	118 0

Sodium and Total Base Calculated.

$$B_2 = \frac{B_1 V_1}{V_3}$$

Where V_3 = Serum volume in 125 cc of treated blood.

HCO_3 and Cl Calculated.

$$A_2 = \frac{A_1 V_1}{V_3}$$

Where A_1 = HCO_3 or Cl found in 1000 cc of untreated serum.

A_2 = " " " calculated in 1000 cc of treated serum.

HCO_3 (Tables II and III).

Bohr's solubility coefficients (3) for cells and serum are used throughout these experiments though it is realized there is a slight inaccuracy according to Hastings' (10) recent paper. $\text{HCO}_3 = \text{CO}_2$ content - H_2CO_3

$$\text{H}_2\text{CO}_3 = \frac{\text{Solubility coefficient } \text{CO}_2 \times p\text{CO}_2}{760}$$

Where $p\text{CO}_2$ = CO_2 tension

Solubility coefficient of CO_2 in serum at 38°C = 0.555×0.975 .

" " " " " cells " 38° " = 0.555×0.81 .

Where 0.555 = solubility coefficient of CO_2 in H_2O at 38°C .

The effect of adding salt or water to whole blood will be noted in the following changes: There is an increase in serum volume or shift of water from cells to serum in all except the two water experiments where cell volume increases. There is an exchange of Cl and HCO_3 across the cell membrane when salts of chloride or carbonate are added to the blood. When chloride is added, Cl penetrates the cells and HCO_3 escapes to the serum; and similarly, on adding carbonate, HCO_3 enters the cells while Cl passes into the serum. When water is added to the blood, there is a shift of both HCO_3 and Cl from cells to serum.

The sodium and total base found in the serum agree in all the experiments within 3.5 m.-eq per liter with that calculated on the assumption that no base traverses the cell membrane. The variation is not constantly above or below the calculated and in most of the experiments is less than 2 m.-eq. In three experiments where equal concentrations of sodium and potassium salt were added, the changes in the distribution of water, CO_2 , and chloride produced by the two salts are practically identical.

DISCUSSION.

There can be little doubt that within the limits of the above experiments the red blood cell membrane has consistently remained impermeable to both sodium and potassium. Inasmuch as the most extreme changes in total base concentration found in health or disease are well within the limits of the changes produced in the present experiments (see Table IV) it is fair to assume that in the human body the red blood cell membrane remains impermeable to the cations sodium and potassium throughout all the equilibrium readjustments of the blood.

The assumption is further supported, as was mentioned in the introduction, by nearly all those workers who have made observations on the permeability of the red blood cells after exposing blood to varying tensions of CO_2 . Gurber (6), in 1895, was the first to point out the apparent impermeability of red blood cells to cations. He reported that though the serum appeared to become more alkaline when blood was saturated with CO_2 , he was able to demonstrate by ash analyses and estimation of serum volume changes that there was no actual increase in serum base content, but that sufficient Cl had passed into the cells to account for the

increase in BHCO_3 . 2 years later, Koeppe (12) tried the effect of passing CO_2 through a mixture of washed cells in isotonic glucose solution, and reported that he was unable to recover any titratable alkali from the glucose solution. Hamburger in his splendid monograph (7) (1902), in which he reported a great many red blood cell permeability experiments with a variety of salts, apparently believed at that time that the cells were impermeable to cations. More recently, Henderson, McLean, and Murray (11) and Doisy and Eaton (5) have reported from data obtained in CO_2 saturation experiments that the electrolyte readjustments can be accounted for almost entirely by transfer of water and anions across the cell membrane. In 1917, Van Slyke and Cullen (18) stated that some base probably crossed the cell membrane when blood was saturated with CO_2 ; but in 1921, Van Slyke (19) was of the opinion that the

TABLE IV.

Comparison of Limits of Concentration Change Produced in Experiments with Those Found in Human Disease in This Clinic

	Serum CO_2		Serum Cl		Total base	
	Low	High	Low	High	Low	High
	m -eq per l	m -eq per l	m -eq per l	m -eq per l	m -eq per l	m -eq per l
Experiments	17 4	37 5	80 4	142 3	116 8	195 5
Human disease	2 4	54 0	63 3	126 5	125 0	180 0

cation shift must at most be slight compared with the anion transfer. Collip (4), using the same method of equilibrating blood at various CO_2 tensions, concluded that both acid and base could cross the cell membranes, but that the permeability to base only became noticeable when using CO_2 tensions considerably beyond the physiological range. The evidence that is derived from the CO_2 saturation experiments, in which the changes in CO_2 tension do not exceed the limits found in the human body, is overwhelmingly in favor of the impermeability of the red blood cells to cations.

But Hamburger and Bubonovic (1911) (8), and Hamburger (1916) (9) reported that the red blood cell membrane was permeable to both sodium and potassium when they added small amounts of sodium or potassium chloride or water to blood. They used an amount of salt equivalent to 0.2 gm. per 100 cc. of serum and an amount of water equivalent to 10 per cent of the serum

volume. It will be noted that considerably larger amounts of salt and water are added in the present experiments and that in spite of the consequent greater disturbance of electrolyte equilibrium, no cations appear to traverse the cell membrane.

In trying to explain these discordant findings, the methods used by Hamburger and Bubonovic were carefully reviewed. In both papers, permeability to base was ascertained by sodium and potassium estimations in cells and serum. Potassium was determined either as cobalti-nitrite or chloroplatinate and sodium was calculated by difference after the gravimetric determination of the total $KCl + NaCl$ content. In the earlier paper, 900 cc. of beef blood were used for each experiment and the chloroplatinate method was used. After a preliminary saturation of the blood with 5 volumes per cent of CO_2 very little precaution seems to have been taken against loss of CO_2 between the time cell volume was determined and the cells and serum were separated. Further, the salt or water was added to serum that had been decanted from the sedimented cells into a second vessel. This treated serum was then poured back into the original container without, apparently, taking any care against loss of CO_2 or serum in the transfer. In the second paper, the potassium was determined by a volumetric estimation of the potassium cobalti-nitrite precipitate. Although individual determinations show excellent duplicate agreement, it is conceivable, since potassium cobalti-nitrite is known (17) to be an unstable compound, that some of the variation in potassium found might be accounted for by changes in the serum in the different experiments. It is worth mentioning, also, that in all the experiments the sodium and potassium are reported to have crossed the membrane in opposite directions. Since sodium was determined by difference, any analytical error in the potassium would cause an opposite error in the estimation of sodium. The actual shift of base across the membrane is reported as being tremendous. In both series of experiments, when the serum $NaCl$ content was increased by 25 per cent, approximately 20 to 30 m.-eq. per liter of sodium entered the cells while the serum potassium increased about 2 m.-eq. When an amount of water equal to 10 per cent of the serum volume was added, there was again a loss of about 15 m.-eq. per liter of sodium from the serum with an increase of 1.5 m.-eq. per liter of potassium. In the experiments,

therefore, where the NaCl was added, over 80 per cent of this sodium is reported to have entered the cells. In spite of this, the cells are shown to have shrunk to about 88 per cent of their original volume. These findings seem quite unreasonable. Some of the incongruities may be accounted for by the changes in cell volume attendant upon an unguarded loss of CO₂. Finally, it is quite inconceivable how these workers were able to obtain accurate CO₂ equilibrations while using such large quantities of blood.

The disadvantage of using such small quantities of blood as were taken for the present experiments appears to be more than compensated for by the relatively perfect CO₂ saturation and by the very strict precautions against any loss of CO₂. The sodium and total base methods were found satisfactory. The probable maximal error of either method is ± 3 m.-eq. per liter. Inasmuch as the "calculated" sodium or total base was estimated from data subject to the same error, a difference between the "found" and "calculated" might conceivably be as large as 6 m.-eq. but in most instances the error of the method could not account for a difference greater than 3 to 4 m.-eq. In Experiment 5 where the greatest disturbance of electrolyte equilibrium was produced 6 to 8 per cent of the added base might have crossed the cell membrane undetected. However, in five of the eight present experiments, the difference between found and calculated is less than 2 m.-eq. per liter and the possible undetected transfer of base varies from 1 to 5 per cent. This error is insignificant in comparison with any similar experiments that have been reported.

If the cell membrane were permeable to both sodium and potassium, it is difficult to explain the almost exclusive separation of potassium within the cells and sodium in the serum. If this peculiar distribution were due to a selective permeability on the part of the membrane for potassium, it is quite incredible that the electrolyte changes in the first three experiments would show such close agreement when equimolar amounts of a similar potassium and sodium salt were added. For these reasons, it would appear that red blood cells *in vitro* are impermeable to both sodium and potassium and that probably when they are in circulation they remain impermeable to these cations. At present, it seems impossible to explain the peculiar distribution of sodium and potassium between cells and serum on any basis which assumes a trans-

fer of these cations across the membrane of red blood cells in circulation.

It is interesting to note that in the last five experiments where the serum protein determinations are believed to be reliable there is a close agreement between the ratio of serum volume change based on the hematocrit determinations and on the serum protein determinations (see Table V).

SUMMARY.

It was believed on theoretical grounds that the red blood cells were impermeable to the cations sodium and potassium within the limits of total base change found in human disease. CO₂ saturation experiments support this belief, for in the reestablishment of electrolyte equilibrium, the changes can practically all be ac-

TABLE V
Comparison of Ratios of Serum Volume Change as Determined by Hematocrit and Serum Protein

Experiment No	$\frac{\text{Serum volume}_1}{\text{Serum volume}_2}$	$\frac{\text{Serum protein}_2}{\text{Serum protein}_1}$
4	94 0	93 8
5	95 2	94 6
6	92 8	93 8
8	76 9	78 2

counted for by the transfer simply of water and anions across the cell membrane. But Hamburger found that when he added Na or K salts or water to blood, both basic and acid ions appeared to traverse the cell membrane. In this paper are reported the results obtained on repeating some of Hamburger's experiments. Human blood equilibrated at 40 mm. of CO₂ in air at 38°C. was used. Sodium and potassium salts of chloride and carbonate were added in concentrations approximating the extremes found in human disease. To other samples sufficient water was added to cause a 30 per cent dilution of the serum. Complete electrolyte determinations, including sodium and total base, were done and the transfer of H₂O, CO₂, and Cl across the cell membrane was demonstrated. In no instance was there any evidence of a shift of base across the cell membrane, and when equimolar amounts of a like Na and K salt were added to specimens of the same blood, the

electrolyte changes were practically identical. Hamburger's experiments have been reviewed and certain criticisms offered. It is believed that the experiments presented very definitely indicate an impermeability of the red blood cell to the cations sodium and potassium.

CONCLUSIONS.

1. The human red blood cells appear to be impermeable to the cations sodium and potassium during electrolyte readjustments caused by either the addition of sodium or potassium salts of chloride or carbonate or the addition of water. Equilibrium is reestablished, apparently, by the transfer of H_2O , CO_2 , and Cl across the cell membrane.

2. When equimolar amounts of a similar sodium and potassium salt are added to blood the changes in the distribution of water, CO_2 , and Cl are quantitatively practically identical in the two analyses.

3. No explanation is offered for the extraordinary distribution of potassium and sodium between the cells and their surrounding fluid for the cell membrane is apparently equally impervious to both of these cations.

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THE BEHAVIOR OF THE PROLAMINS IN MIXED SOLVENTS.

III. THE DENATURATION OF WHEAT GLIADIN.

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Dill and Alsberg (1) have shown that gliadin, though prepared by extraction with hot aqueous alcohol, may undergo an irreversible loss of solubility if allowed to stand for some time in contact with aqueous alcohol. To determine what rôle concentration of alcohol and temperature play in this alteration of gliadin is the purpose of the investigation herein reported.

The method used to detect alteration of gliadin was to determine the "critical peptization temperature" of its solutions. By "critical peptization temperature" is meant the temperature at which the first signs of turbidity appear in a solution of gliadin in alcohol-water when it is cooled slowly. It will hereinafter be designated as C.P.T. It was assumed that if a given treatment changed the C.P.T., this could be taken to indicate alteration of the gliadin.

The C.P.T. was determined by the method used by Dill and Alsberg. 2½ per cent solutions of gliadin were prepared in 20, 40, 50, 60, 70, and 80 volume per cent alcohol. Some experiments were also made with solutions in 90 per cent alcohol but here only 0.5 per cent solutions of gliadin could be used because of low solubility in this strength of alcohol. All the solutions were quite clear at room temperature except those in 90, 80, 30, and 20 per cent alcohol. Hence in these cases in the intervals between the tests the solutions held at the lower temperatures contained the gliadin in part undissolved. To obtain clear solutions in 90 per cent alcohol required a temperature of 65°C., in 80 per cent alcohol 55°C., in 20 per cent alcohol 45°C.

Small quantities of the solutions thus prepared were sealed hermetically in small glass tubes about 0.6×5 cm. in size. The tubes were then at once warmed to a temperature at which all the solutions were clear and thereupon slowly cooled. The temperatures at which the first distinct opalescence appeared in the different tubes were noted. These are the C.P.T. at 0 days. The tubes were then divided into a number of sets each containing at least two tubes for each alcohol-water strength. The different sets were then held at different temperatures ranging from 4–80°C. After the lapse of some days the C.P.T. was again determined. These determinations were then repeated after several further intervals of holding at the same temperature.

Table I gives the results of such an experiment made with gliadin Preparation 3A described in the paper of Dill and Alsberg. In this series the strongest alcohol used as solvent was 70 per cent and the highest holding temperature 58°C. It will be noted that the results show much irregularity. This is equally true for each of the other series made with other gliadin preparations which it has not seemed worth while to record here in detail. In each of the series, however, one common trend is so striking as to be beyond doubt. It is that in very weak alcohol, notably in 20 and 30 per cent, the C.P.T. is raised at all holding temperatures, even at 4°C. This is the only new fact this investigation has brought out with certainty but it is one, as will be pointed out, of considerable significance. It is possible that the warming to 45°C required to clear the solution for each determination may account for some of the alteration in 20 per cent alcohol.

However, the data recorded in the table as well as other similar ones not here recorded are suggestive. Thus there seems to be a tendency in a number of instances and especially in 70 per cent alcohol for the C.P.T. to become lower with time. It is not possible at the present time to offer an explanation, though it is perhaps the result of some hydrolysis which tends to lower the C.P.T. (2). The data are not sufficiently regular to warrant further analysis. Indeed, the writers did not succeed in obtaining a single series in which there were no irregularities, though the general effect at very low and very high alcohol concentrations showed the same trend in all series. Experiments with 80 per cent alcohol as solvent showed a raising of the C.P.T. as compared with 70 per

cent alcohol. This is in harmony with the observations of Dill and Alsberg. The experiments with 90 per cent alcohol were unsatisfactory because so small a percentage of gliadin was dissolved.

TABLE I.

Effect of Heat and Alcoholic Concentration upon Critical Peptization Temperature of Gliadin 3A.

Alcoholic strength of solvent by volume	Holding temperature	CPT at 0 days	CPT at 4 days	CPT at 9 days	CPT at 15 days
<i>per cent</i>	<i>°C</i>	<i>°C</i>	<i>°C</i>	<i>°C</i>	<i>°C</i>
20	4	33 00	39 0	39 00	36 5
20	25	33 00	45 0	42 5	45 0
20	37	33 00	39 0	39 0	39 5
20	58	33 00	42 0	42 0	42 0
30	4	26 00	37 0	38 0	27 5
30	25	26 00	36 5	36 5	29 0
30	37	26 00	36 0	34 0	27 5
30	58	26 00	36 0	32 0	33 0
40	4	16 00	16 5	16 0	16 5
40	25	16 00	16 0	15 5	15 5
40	37	16 00	16 0	16 5	17 0
40	58	16 00	17 0	17 5	19 0
50	4	7 5	7 5	7 5	6.5
50	25	7 5	8 0	7 0	7 5
50	37	7 5	7 5	7 0	7 5
50	58	7 5	8 0	9 0	9 5
60	4	2 0	2 0	2 0	2 0
60	25	2 0	2 0	2 0	2 5
60	37	2 0	2 5	2 5	3 0
60	58	2 0	2.5	3 0	4 0
70	4	10 0	9 0	7 5	7 5
70	25	10 0	9 0	7 5	7 5
70	37	10.0	9 0	7 5	8.0
70	58	10.0	9 0	9 0	10.0

The observations here reported are not without analogues. It may seem strange that a protein should be changed at so low a temperature as 4°C., yet Homer (3) and others have presented evidence that serum albumin is altered by a temperature as low as

40°C. It has of course long been known that globulins lose in solubility on standing in water. It may seem strange that altered gliadin which has been separated by cooling is again so readily dissolved by merely raising the temperature. For this, too, there are precedents. Heating serum, as is done in the concentration of antitoxin, can be conducted in such a way that the proteins are altered without producing opalescence. From such heated serum, protein may be precipitated at a lesser salt concentration than from unheated serum. Much of the protein thus precipitated can again be dissolved in water.

From the demonstration herein presented that gliadin is so readily altered by contact with weak alcohol, it follows that in the preparation of gliadin such contact should be avoided. Indeed the writers have found in preparing gliadin that evaporating solutions in 70 per cent alcohol too far reduces yields greatly and gives preparations with very high C.P.T., even when the concentration is carried on under diminished pressure at a temperature as low as 50°C.

It may well be, moreover, that these observations have some important practical bearings. It is well known that the manner of curing wheat has a decided influence upon the baking quality of its gluten. It is possible that if the curing of the wheat berry goes on slowly, as in damp weather, so that the berry dries out gradually and the gliadin in its endosperm remains for a long time in contact with free water, the colloidal state of the gliadin is affected in a manner different from that in which it is affected when wheat is rapidly cured and soon becomes dry. Moreover, the temperature of the weather may also play a part in the process. Perhaps changes in gliadin like those here recorded may play a rôle in the development of the gluten during the doughing process and in the maturing of the dough during panary fermentation. In some of the processes of making bread or crackers the fermentation may be prolonged for many hours. These are problems for future investigation.

For the observation that the C.P.T. is largely independent of the gliadin concentration (Dill and Alsberg) there are also analogues in the literature. For example, Guthrie (4) has shown that opalescence appears in solutions of triethylamine in water at almost

the same critical temperature in concentrations ranging from 15 to 50 per cent. To make a comparison between gliadin solutions and solutions near their critical points is not far-fetched. Konovalow (5) has shown that colloidal solutions and solutions at the critical point resemble one another greatly. In the present connection, it is of interest that to change the concentration of either requires almost no work and that in both the slightest of causes is adequate to produce local disturbance of the homogeneity of the solution. Moreover, Zsigmondy (6) has pointed out that certain ternary systems containing colloid might be expected to behave like binary mixtures of liquids at or near the critical point. He even suggests that the phase that separates varies in composition according to circumstances, as is the case for the phases of a binary solution at the critical temperature. He has proposed the term "vacillon" for the individual particle of such a disperse phase.

Finally the observation made by Dill and Alsberg, and confirmed by the writers, that different preparations of gliadin may have C.P.T. differing in certain instances by as much as 10°C., raises the question whether or not gliadin, as it is obtained by hot extraction with aqueous alcohol, is not already denatured. It is entirely conceivable that it is then in a state different from its original state in the endosperm of the wheat berry. Possibly alcohol denatures it, the denatured product being peptized by mixtures of alcohol and water. The fact that gelatin resembles gliadin in so many ways, for example in its solubility in aqueous alcohol (7), indicates that, pending direct evidence, perhaps a mental reservation is called for in regard to the native character of gliadin, for gelatin is most certainly not an undenatured protein, since in its preparation high temperatures or acids or both are used.

SUMMARY.

Weak alcohol, 20 and 30 volume per cent, acting upon gliadin at low temperatures, alters it in respect to solubility. 80 per cent alcohol also alters it. At intermediate concentrations of alcohol very little alteration takes place even at higher temperature.

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THE ADSORPTION OF CAROTIN BY DIFFERENT CHARCOALS AND INORGANIC SALTS.

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In the course of an investigation on the differentiation of carotin from vitamin A, Stephenson (1920) found that the carotin of butter fat could be completely removed by agitating its solution in petroleum ether with 2.7 gm. of birch charcoal per 100 gm. of butter fat for 4 hours. Palmer (1922, p. 270), who experimented with a number of different charcoals without being able to confirm this observation, suggested that Stephenson's findings might be repeated. Using the most effective available carbon in adsorption experiments in which the possibility of oxidation had been removed, Palmer (1922, p. 219) found that at least 5 times (12.5 gm.) the amount of charcoal used by Stephenson was required to adsorb the carotin completely. This difference in experience appeared so marked that it seemed worth while to investigate the adsorptive power of a series of charcoals for their capacity in removing the carotin of butter fat.

A large number of charcoals is obtainable, made by different processes and from many sources, and varying widely in composition and adsorptive capacity (*vide* Mellor, 1924). Much of the conflicting evidence on adsorption phenomena with charcoals is attributable to these causes and appears to be the explanation in the present case. The evidence, for example, on specific adsorption by charcoals in general is still inconclusive, although in many particular cases successful applications of the principle are known. Cole's test for lactose (1920, p. 313) is thus based upon the specific action of Merck's blood charcoal for lactose in the presence of glucose.

In the work recorded here, it was found that different charcoals vary to a surprising extent in their capacity for adsorbing carotin, a result which would appear to account for Palmer's failure to repeat Stephenson's earlier finding. It is also significant that Stephenson mentions the fact that of three samples of birch charcoal, only one exhibited the adsorptive effect described. As these three adsorbents were prepared by the Chemical Warfare Department (*vide* Cole, 1920, p 394), it is probable that the one employed by Stephenson had been subjected to activation by heat treatment, a phenomenon which has been closely studied by Philip and his coworkers (1919, 1920). The best adsorbent of the series investigated was the vegetable charcoal norit, which was found to be completely effective at the rate of 3.5 to 100 gm. of butter fat. On the other hand, as much as 40 gm. of charcoals such as coconut, willow, and varieties of birch were practically useless for this purpose. The figures for the ash content of the charcoals fail to reveal any correlation with their adsorptive capacity. It is perhaps noteworthy that iron was found in all the ashes.

From Tswett's pioneer work (1906) on the adsorptive properties of the chromolipoids, it was known that certain inorganic salts can also adsorb carotin from its petroleum ether solution. A considerable number of inorganic salts was therefore investigated, most of which proved to be ineffective. Red antimony trisulfide, however, was found to be unexpectedly active, while the black modification was totally inactive.

In discussing Stephenson's work on carotin, McCollum (1922) appears to misinterpret her findings. He states, from her results, that "an impure preparation of carotin, which did not behave like a source of fat-soluble A when fed as such, acquired the properties of this dietary factor when it was dissolved in palm-kernel oil previously tested and found ineffective for the stimulation of growth." However, the impure source of carotin fed by Stephenson, in both her experiments exerted a definite growth response indicating the presence of vitamin A. The conclusion of McCollum, therefore, apart from the disproof afforded by the recent work of Drummond, Channon, and Coward (1925), that vitamin A may not be absorbable unless carried by small amounts of fat, is not justified.

EXPERIMENTAL.

A number of trial experiments was carried out using a range of charcoals so as to arrive at some idea as to the adsorptive capacity of each for butter fat carotin. A sample of English dairy butter of good quality and deep yellow in color was used. This was first melted, the water present separated off, and the butter fat filtered through muslin. 50 gm. portions were immediately weighed off. It was observed that butter fat is readily bleached by exposure to light and air for about 3 weeks, or by heat. Colorimetric estimation of the carotin present indicated an approximate value of 0.0042 per cent for the butter fat.¹ The 50 gm. portions were now placed in wide mouthed bottles provided with tight fitting glass stoppers and 200 cc. of petroleum ether (b.p. 50–60°C.) added to each. By bubbling a stream of nitrogen for 2 minutes through the solutions, it was considered that the possibility of oxidation as a complicating factor in the removal of the carotin (*cf.* Steenbock, Sell, and Buell, 1921), had been removed.

From these preliminary results, further work, with the same concentration of butter fat, gave approximate figures for the least quantity of any charcoal required to decolorize the fat. All the solutions were shaken mechanically at constant speed for a period of 4 hours. It was observed that most of the pigment was adsorbed after 2 hours shaking, and treatment for a further 2 hours usually succeeded in removing the remainder.

Most of the charcoals were already in a fine state of division when received; the remainder were reduced in the mortar until they passed a sieve of $\frac{1}{32}$ inch mesh. The results are summarized in Table I. From this table it is seen that there are striking differences in the adsorptive power of different charcoals for the carotin of butter fat in petroleum ether solution.

¹ In reviewing Steenbock's theory of the possible identity of vitamin A with one of the carotinoid pigments, Palmer and Kennedy (1921) dealt with another experiment of Miss Stephenson (1920) in which pure crystalline carotin dissolved in palm kernel oil, was fed to rats as the sole source of vitamin A, a negative result being obtained. These investigators pointed out that Stephenson's omission to describe the composition of her basal diet rendered difficult their calculation of the exact percentage of carotin fed, but assumed that the diet contained 15 per cent of fat. This assumption was correct.

A qualitative examination of the ash of each charcoal was carried out, the results of which are combined in Table II.

According to Merck's standards (1907), animal charcoal should contain not more than 10 per cent ash, in which copper should be absent and calcium and iron present only in insignificant amount. Certain inorganic salts can adsorb carotin; thus Tswett (1906) found that finely divided mercuric chloride, calcium chloride, and

TABLE I
Adsorptive Capacity of Different Charcoals for Carotin

Sample No	Description	Amount required to decolorize 100 gm butter fat.
		<i>gm</i>
1	Norit (vegetable)	3 5
2	Blood	8 0
3	"	8 0
4	Merck (granulated medicinal)	13 0
5	Merck (ordinary medicinal)	14 0
6	Birch (dust)	24 0
7*	" (stock)	More than 8 0
8*	" (activated)	" " 8 0
9*	" "	" " 8 0
10	" (coarse)	" " 40 0
11	Decolorizer	" " 40 0
12	Coconut	" " 40 0
13	Willow	" " 40 0
14	Fruit shell	" " 40 0

* Samples 7 to 9 were obtained from Prof J C Philip who kindly supplied the following information in regard to their preparation Sample 7 was a stock birch charcoal from which Samples 8 and 9 were prepared by heating at 800°C in a limited supply of oxygen and had thereby become activated (Philip, 1919; Philip and Jarman, 1924) These samples, which were received as $\frac{1}{4}$ inch cubes, were reduced in the mortar so as to pass a sieve of $\frac{3}{32}$ inch mesh.

lead sulfide are effective adsorbents. Repeating this observation on butter fat in petroleum ether, calcium chloride was found to be more effective than mercuric chloride. Lead sulfide was not tried but a number of other sulfides were investigated adopting a uniform procedure. They were studied under the same conditions as for the charcoals, at the rate of 40 gm. of sulfide to 100 gm. of butter fat. Red antimony trisulfide (40 gm.) proved to be the

most active, while the sulfides of calcium, cadmium, and copper exhibited some small capacity. Black antimony trisulfide, arsenic trisulfide, zinc sulfide, and ferrous sulfide were all found to be inactive. The capacity of certain sulfides for adsorbing carotin is thus not a general property of this class of compounds.

A number of other inorganic compounds was investigated most of which were without effect in adsorbing carotin. These included dialyzed iron, kaolin, kieselguhr, magnesium silicate, calcium phosphate, calcium oxide, barium oxide, the red and yellow forms

TABLE II
Composition of the Ash.

Sample No	Description	Ash	Ions found
		<i>per cent</i>	
1	Norit (vegetable)	5 27	Ca, P, Fe, Mg, Si
2	Blood	0 62	Fe, Ca (trace)
3	"	1 78	Ca, Fe
4	Merck (granulated medicinal)	25 24	" Mg, Fe
5	Merck (ordinary medicinal)	4 31	" P, Fe (trace)
6	Birch (dust).	7 27	" " Mg
7*	" (stock)	1 13	" Fe, Mg, P (trace).
8*	" (activated)	1 49	" " " " "
9*	" "	2 15	" " " (trace)
10	" (coarse)	3 59	" " P, Mg (trace).
11	Decolorizer	76 78	" P, Mg, Fe "
12	Coconut	1 62	P, Fe, Ca (trace)
13	Willow	13 19	Ca, P, Mg, Fe, K, Si.
14	Fruit shell	12 53	" Mg, Fe, Si, P (trace).

* See footnote below Table I

of mercuric oxide, calcium carbonate, and a sample of powdered slate in a very fine state of division from deposits on beams in the Penrhynn quarries, North Wales. The latter has been found to be a satisfactory adsorbent for bacterial toxins by Professor J. M. Beattie of the University of Liverpool (1926) and it was thought to be of interest to test its capacity in adsorbing carotin. It was found to be ineffective.

I am indebted to Sir F. Gowland Hopkins for his interest and support.

SUMMARY.

1. The discrepancy between Stephenson's and Palmer's figures for the amount of charcoal required to adsorb butter fat carotin is explained by the fact that different charcoals vary widely in their adsorptive capacity towards this pigment.

2. No clear relationship could be traced between the adsorptive power of a charcoal and its ash content.

3. Inorganic compounds as a class are not good adsorbents of butter fat carotin, but red antimony trisulfide is effective while the black variety is inactive.

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THE NUCLEOTIDES OF TRITICONUCLEIC ACID.

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(Received for publication, March 28, 1927)

Triticonucleic acid was isolated from wheat embryo by Osborne and Harris (1) in 1902. With 750 gm. of the substance in their possession they made a thorough study of its properties and some of its hydrolytic products. Among the latter they found adenine, guanine, pentose, and uracil and suggested the identity of triticonucleic nucleic acid and yeast nucleic acid. In 1910, Levene and La Forge (2) improved the method of preparing triticonucleic acid and found that on hydrolysis with ammonia in an autoclave, at 145°, it was decomposed into the nucleosides and phosphoric acid but they only isolated adenosine, guanosine, and cytidine. They also showed that the pentose is identical with the one obtainable from yeast nucleic acid. Read and Tottingham (3), in 1917, prepared guanine nucleotide, the only nucleotide known at that time, and what they believed to be adenine-uracil dinucleotide. The guanine nucleotide was identical with the guanine nucleotide obtained from yeast nucleic acid. The nucleoside, uridine, prepared by them from the adenine-uracil dinucleotide was identical with the known uridine. As Jones (4) has pointed out there is little doubt that triticonucleic acid and yeast nucleic acid are identical.

In view of the recent work of Jones and Perkins (5) we decided to investigate the *nucleotides* of triticonucleic acid, since only one of them, namely guanine nucleotide, has been prepared and that one not in crystalline form. With this object in view, more than 100 gm. of triticonucleic acid was prepared from wheat embryo from which all four nucleotides have been isolated and identified.

EXPERIMENTAL.

Triticonucleic acid was prepared from wheat embryo according to the method described by Levene and La Forge (2). In order to hydrolyze triticonucleic acid into its nucleotides the method of Steudel and Peiser (6), as modified by Jones and Perkins (5), was used.¹ Two samples of 50 gm. each were used. One was allowed to digest in a dilute solution of sodium hydroxide at the room temperature for 36 hours while the other was allowed to digest 2 weeks. The two samples were carried through side by side and showed not the slightest variation from each other.

A modification of the method of our predecessors was to evaporate the filtrate from the lead salts to a very small volume at a low temperature (40–50°) and add 4 to 5 volumes of 95 per cent alcohol.² The lead salts of the nucleotides are insoluble, and when taken through the usual procedure, each of the two experiments gave 4 gm. of mixed nucleotide material. The only other point where we differed from our predecessors was that we evaporated the alcohol used in the hardening of the mixed nucleotides. Here we obtained an additional 3 gm. in each experiment, the greater part of which was undoubtedly uracil nucleotide. The total mixed nucleotides recovered in each case was 44 gm. They were separated into a *guanine fraction* and an *adenine fraction* by the usual procedure.

¹ At the time this work was being done we did not know that the hydrolysis could be accomplished by the use of strong ammonia at the room temperature as has been shown recently by one of us and Jones (7). When sodium hydroxide is used as the hydrolytic agent there are several possibilities for the loss of uracil nucleotide. In the first place the amorphous lead salt of uracil nucleotide has a specifically great solubility in sodium acetate solutions, and secondly, the uracil nucleotide itself is the most soluble of all the nucleotides in alcohol. This undoubtedly accounts for the loss of uracil nucleotide by Jones and Perkins (5) and by Calvery (8), who obtained the same results as Jones and Perkins when he hydrolyzed nucleic acid with sodium carbonate. From the alcohol used to harden the nucleotides Jones and Perkins found some material which gave a brucine salt having the composition of the brucine salt of uracil nucleotide but they did not credit it with any significance since the amount they should have found was many times more.

² This modification is unnecessary when nucleic acid is hydrolyzed with strong ammonia at the room temperature since the excess of ammonia can be removed by evaporation.

Guanine Fraction.—Crystalline guanine nucleotide was prepared according to the method of Buell and Perkins (9).

Micro-Dumas-Pregl for nitrogen ³

I. 3.333 mg. gave 0.510 cc. N at 23° and 754 mm.

II. 4.781 " " 0.732 " " " 23° " 754 "

	Calculated for guanine nucleotide	I Found	II
N... ..	17.54	17.51	17.52

Adenine Fraction.—The nucleotides of the adenine fraction were converted into their brucine salts by the addition of a hot saturated solution of brucine, in 95 per cent alcohol, to a hot concentrated solution of the nucleotides in water. The brucine salts obtained were recrystallized nine times from 35 per cent alcohol.

Crystallization No	Analysis of crystals for nitrogen ⁴ per cent
1	8.16
2	7.96
3	7.87
4	7.59
5	7.28
6	6.89
7	6.75
8	6.71
9	6.81
Required for.	
Cytosine nucleotide	7.92
Uraci "	6.79

Adenine Nucleotide.—Most of the adenine nucleotide crystallized out of the adenine fraction when it was evaporated to a small volume before making the brucine salts. It was recrystallized from water and analyzed.

³ The analyses of the products obtained in this work were done by one of us under the supervision of Dr. O. Wintersteiner, Fellow of the Rockefeller Foundation, from Professor Pregl's laboratory at Graz.

⁴ Since the brucine salt of adenine nucleotide has a much higher nitrogen per cent than the other nucleotides there may be a question as to why the first analysis shows such a low nitrogen percentage. It is due to the fact that before the brucine salts were made most of the adenine nucleotide had crystallized.

Micro-Dumas-Pregl for nitrogen

I. 2 606 mg gave 0 431 cc N at 24° and 759 mm.

II. 3 205 " " 0 534 " " " 24° " 759 "

	Calculated for adenine nucleotide	I	Found	II.
N..	19 18	18 98		19 12

Cytosine Nucleotide.—The brucine salts obtained by evaporation of the mother liquors from Crystallizations 3, 4, and 5 in each experiment were combined and taken through the usual process for recovery of the free nucleotide. On concentration of the clear solution crystalline cytosine nucleotide separated. It was re-crystallized once from hot water and analyzed.

Micro-Dumas-Pregl for nitrogen

I. 4 091 mg gave 0 464 cc N at 21° and 752 mm.

II. 3 305 " " 0 376 " " " 21° " 752 "

	Calculated for cytosine nucleotide	I	Found	II
N..	13 00	13 03		13 07

Uracil Nucleotide.—The final residues of brucine salts were combined and the crystalline ammonium salt, the crystalline lead salt, and crystalline uracil nucleotide were prepared according to the methods described by Levene (10). The uracil nucleotide was analyzed with the following results.

Micro-Dumas-Pregl for nitrogen

I 4 578 mg gave 0 347 cc N at 24° and 758 mm.

II. 3 365 " " 0 254 " " " 23° " 754 "

	Calculated for uracil nucleotide	I	Found.	II.
N	8 64	8 68		8 63

DISCUSSION AND SUMMARY.

Four nucleotides have been prepared from triticonucleic acid which are identical with the four nucleotides that have been obtained from yeast nucleic acid. This lends further evidence to the belief that triticonucleic acid is identical with yeast nucleic acid.

A modification of the procedure of Jones and Perkins (5) has been used, which, we believe, will always enable one to recover all four nucleotides when present.

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STUDIES ON PENTOSE METABOLISM.

II. A MICRO METHOD FOR THE DETERMINATION OF PENTOSE AND PENTOSANS.

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(Received for publication, March 29, 1927)

The decomposition of pentoses or pentosans by means of hydrochloric acid has long been the most extensively used method for transforming these substances into furfural. The subsequent determination of the furfural has been done mainly by precipitating the furfural as phloroglucide and weighing the product according to the directions of Krober, as modified by Tollens, and at present the method of the Association of Official Agricultural Chemists (1). For the literature on this subject the papers of Pervier and Gortner (2) are referred to.¹

The fact that the above method is not adaptable as a micro method, is very time-consuming, yields a phloroglucide of inconstant composition (4), as well as that it is not specific for furfural (5), has made it inefficient for many determinations, particularly those of a biological nature where only small amounts of material or those low in pentose percentage are in question.

Since the development of the steam distillation procedure by Pervier and Gortner and by work in this laboratory, for transforming pentoses into furfural and the colorimetric method of Youngburg and Pucher (6) for the determination of furfural, it seemed that it would be desirable if in the distillation the volatile hydrochloric acid could be replaced by some other reagent which would not itself appear in the distillate, thus obtaining the furfural in a

¹ The colorimetric method of McCance (3) appeared after the work of this paper was completed. The knowledge that refluxing with strong acid such as HCl destroys some furfural must make the method of McCance an approximate one only.

smaller volume, devoid of acidity, and therefore suitable for the direct colorimetric determination

With this object in view the writer has searched for a suitable reagent to replace the HCl in distilling. The literature shows that the following reagents have been employed at one time or another for the decomposition of pentoses into furfural: pyrolusite and sulfuric acid, sulfuric acid, solution of zinc chloride, glacial acetic acid, phosphoric acid, and hydrochloric acid. Only the last seems to have stood the test of time in this respect and its superiority has seemed apparent. The writer, however, has tried all of the above and in addition has used glycerol. The results obtained have eliminated all except phosphoric acid as a competitor of hydrochloric acid for use in this method.

Mann, Kruger, and Tollens (7) in 1896 reported the use of phosphoric acid as follows: "Ebensowenig Erfolg hatten wir, als wir Xylose statt mit Salzsäure mit Phosphorsäure destillierten, denn es bildete sich erheblich Humin, und das entstandene Furfural betrug nicht mehr, sondern etwas weniger als sich mit Salzsäure bildet. (Sehe das Nähere in der Dissertation.)"

The Dissertation of Dr. Kruger is not available. With the use of steam distillation the phosphoric acid method of decomposition might obviously give more satisfactory results because there is efficient means for removing the furfural from the destructive effect of the strong acid. This has been found to be correct and it also has been found that phosphoric acid liberates furfural more rapidly than does hydrochloric acid! There is no loss of furfural, recovery being 100 per cent upon a pure furfural solution. The experimental conditions have been worked out by which a maximum yield of furfural is obtained and are implied in the method which follows.

Method.

Special reagents required are:

1. *Furfural*.—Distil a good grade of furfural, *e.g.* Pfanstiehl, at a pressure of 20 to 30 mm. For most practical work the product from distillation at atmospheric pressure is suitable.

2. *Furfural Standard*.—Dilute 1 cc. of the above furfural up to 500 cc., and dilute 1 cc. of this solution up to 232 cc. 1 cc. = 0.01 mg. of furfural.

3. *Redistilled Aniline*.—It is sufficient to distil under atmospheric pressure, but under reduced pressure an absolutely colorless product is obtained.

The apparatus used is shown in Fig. 1. It is essentially a test-tube *B* (25×150 mm.) containing the reaction mixture and is connected with a steam generator *A* and a condenser. A convenient form of steam generator is a 250 cc. three-necked Pyrex flask, although any other form will do. A small condenser with a water jacket about 6 inches long is convenient, although a larger one will serve the purpose.

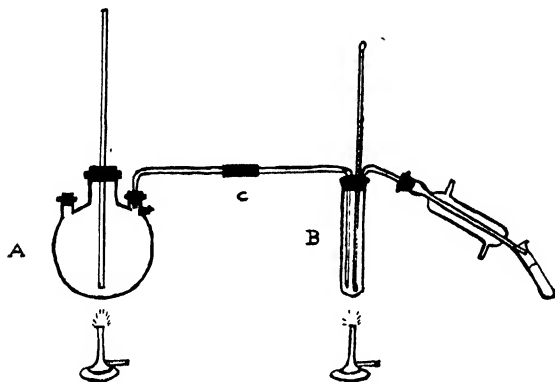


FIG. 1.

Procedure.

Place a quantity of the substance to be determined, calculated to contain between 0.1 and 1.0 mg. of pentose or pentosan, in the test-tube; add about 3 cc. of 85 per cent phosphoric acid and mix. Start the steam generator but do not yet connect at *c*. Connect the test-tube with the condenser and heat the tube with a micro burner until the reaction mixture reaches about 125° . This implies that some water is first distilled off, should much have been introduced with the pentose material. Connect at *c*, causing the influx of a slow current of steam, and distil at about 175° (not exceeding 180°) into test-tube receivers graduated at 10 cc. As each distillate is obtained, transfer exactly 2 cc. to another test-tube containing 0.25 cc. of aniline; add 2 cc. of glacial acetic

acid and set aside for 30 seconds for color development. When no color develops in subsequent tubes, cease distillation and consolidate 2 cc. (or more) aliquots from each of the distillates which give color

Determine furfural colorimetrically in the consolidated aliquots as follows: To one 10 cc graduated tube add 5 cc. of the consolidated distillate and to another similar tube 5 cc. of the furfural standard (0.05 mg of furfural). Add to each tube 0.5 cc. of aniline and 4 cc of glacial acetic acid. Fill to the mark with water and set aside in a dark or semidark place for 15 minutes. Read in a colorimeter. Readings must not extend over 40 minutes from the time of mixing the reagents.

If the color of the unknown is too deep it may be diluted with a mixture of aniline and acetic acid in the above proportions.

The calculation of furfural will of course depend on the number of distillates consolidated. From the furfural obtained is calculated the pentose yield using the following conversion factors:

As <i>d</i> -xylose:	furfural found	× 1.56
" <i>d</i> -ribose	" "	× 2.00
" <i>l</i> -arabinose		
(or <i>d</i> -arabinose):	" "	× 2.40
" <i>d</i> -lyxose.	" "	× 3.00

In the determination of pentosans the writer believes that the results should be expressed as pentose, the particular pentose depending on the pentosan material source.

DISCUSSION.

The literature on the yield of furfural from pentoses by the use of various reagents already indicated in this paper, particularly by hydrochloric acid, gives varying results for *d*-xylose and for *l*-arabinose, and no figures have been published for *d*-ribose and *d*-lyxose, the remaining members of the naturally occurring pentose group of carbohydrates. Pervier and Gortner in their Table I (2) show that pentose recovery by various investigators on xylose ranges from 87.5 to 100.8 per cent, and on arabinose from 73.4 to 100.8 per cent. This is an unusual variation in analytical results and points to some variable which needs better control. That this variable is the partial destruction of furfural by strong

acid during decomposition of the pentose has lately been elucidated by Pervier and Gortner. They obviate this by removing the furfural as soon as formed, by a current of steam.

It is well known, however, that all of the pentoses are not converted into the theoretical yield of furfural. This is firmly established since the destruction of the furfural formed can now be eliminated by steam distillation. Numerous experiments have been made by the writer to find the conditions under which maximal amounts of furfural are produced from the pentoses. Such yields are shown in Table I and were obtained by the method described in this paper.

Thus only *d*-xylose is fully converted into furfural; *d*-ribose,

TABLE I
Showing Furfural Yield or Pentose Recovery by Phosphoric Acid-Colorimetric Method

Pentose	Furfural yield or pentose recovery
	<i>per cent of theoretical</i>
<i>d</i> -Xylose*	100 0
<i>d</i> -Ribose	78 5
<i>l</i> -Arabinose and <i>d</i> -arabinose	64 9
<i>d</i> -Lyxose	52 2

* Until recently known as *l*-xylose. See Armstrong, E F, Monograph on biochemistry, The carbohydrates and the glucosides, London and New York, 4th edition, 1926, 38.

l- and *d*-arabinose, and *d*-lyxose follow in decreasing amounts of furfural yield. While the recovery for *d*-xylose is 100 per cent of the theoretical value, the figure for *l*-arabinose, 64.9, is less than that found by others (73.4 to 100.8) by the hydrochloric acid method. As previously mentioned, recovery figures for *d*-ribose and *d*-lyxose as well as for *d*-arabinose, have not been published heretofore. It is interesting to find that *d*-arabinose yields the same amount of furfural as *l*-arabinose. At least in this case of enantiomorphic forms the difference in configuration does not affect the furfural yield. This is of some importance because the arabinose in the urine of pentosurians has been found by Neuberg (8) to be the racemic form. In its determination, then, no error will be made because the racemic form is equivalent

TABLE II

Showing Yield of Furfural from Various Substances by Micro Method.

Substance *	Furfural yield per cent
Carbohydrates.	
Maltose	5 71
Dextrose	4 84
Dextrin	3 93
Sucrose	3 52
Inulin.	1 98
Starch (potato)	1 85
Glycogen	1 51
Galactose	1 38
Levulose	1 12
Lactose	1 10
Other substances	
Albumin, egg	0 08
“ serum	0 04
Cholesterol.	None.
Creatinine	“
Cystine	Trace
Casein	0 04
Fibrin	0 05
Glutamic acid	None
Glycuronic acid	About 10 0
Glycocoll	None.
Gelatin	0 02
Gum arabic	8 51
Hemoglobin	0 02
Inosite	None
Mucic acid	0 10
Nucleic acid (from yeast)	10 40
Ouabain (a rhamnose glucoside)	None
Rhamnose	“
Salicin (glucose- <i>o</i> -oxybenzyl alcohol)	2 75
Tyrosine	Trace.
 Dulcitol	 None.
Mannitol	“
Sorbitol	“

* The purest substances obtainable were used In a number of cases purifications were made in this laboratory

to a mixture of *d* and *l* forms and they yield equal amounts of furfural.

It is well known that substances other than pentoses, particularly glycuronic acid, yield furfural when heated with strong acid. The yields from various carbohydrates and from a number of miscellaneous substances by the phosphoric acid-colorimetric method herein described have been determined and are given in Table II.

It can be seen that, of the common carbohydrates, maltose yields the greatest amount of furfural, 5.71 per cent. Lactose yields the least, 1.1 per cent. All yields are appreciable. Rhamnose, a methyl pentose, yields no furfural. Pure glycuronic acid was not available but glycuronic acid esters were extracted from urine according to the method of Neuberg and Schewket (9) and yielded much furfural. Dr. Pucher of this laboratory has found a yield of about 10 per cent from glycuronic acid by the phosphoric acid-colorimetric method. Mann, Kruger, and Tollens (7) found 17.27 per cent by the hydrochloric acid distillation method after checking up reports of 46 per cent by Gunther and 31.4 per cent by de Chalmot.

Substances such as amino acids, proteins, nucleic acid, creatinine, etc., were determined in order to test the availability of the method for the determination of pentose in blood, tissues, urine, etc. From the data obtained it appears that only substances containing a carbohydrate unit in the molecule, excepting methyl pentoses like rhamnose, and glycuronic acid, may yield appreciable amounts of furfural. This is in accord with data in the literature.

In determining pentoses by furfural yield, then, one must take into account the probable presence of interfering substances. Body fluids may yield appreciable furfural from non-pentose sources, such as from glycuronic acid, glycogen, dextrose, and nucleic acid compounds. Under present circumstances, however, the method may be of value in following the course of the metabolism of substances which are predominatingly furfural-yielding.

SUMMARY.

Pentoses may be determined in a micro way by distillation with phosphoric acid and steam. The furfural formed is determined colorimetrically.

Phosphoric acid liberates furfural more rapidly from pentose material than does hydrochloric acid. In general, however, the yield of furfural is not greater than with hydrochloric acid.

The method may be of value in metabolism work and for other purposes.

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SULFUR METABOLISM.

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A quantitative measurement of the oxidation of cystine was recorded as early as 1886, when Goldmann (1) reported that two-thirds of cystine sulfur fed to animals appeared in the urine as sulfate sulfur. In more recent experiments there are indications of a far greater capacity for oxidation on the part of the animal organism than is suggested by Goldmann's data. It seems very likely that differences in oxidation levels reported from different laboratories may be due to some protective chemical reaction, probably a union of the cystine with some other substance, resulting in an effective interference with the normal oxidative processes.

In the cysteine molecule there are three points which may be considered vulnerable (2,3): A, the amino radical, B, the sulfhydryl radical, and C, the carboxyl radical.

This hypothesis was submitted to a series of tests by synthesizing ten suitable cystine and cysteine derivatives and feeding them to rabbits. The animals were kept on a basal ration for at least 3 days before any samples were taken. Urine samples were then submitted to nitrogen and sulfur partitions by well known methods (4). The voluminous data are briefly summarized in Table I. The range of sulfur elimination and oxidation are more readily perceived in Table II.

Cystine sulfur administered by mouth to a rabbit in sulfur equilibrium promptly increases the total sulfur of the urine. This increase is assumed to come directly from the sulfur compound ingested and is listed under the caption "Cystine sulfur output." This is also tabulated as per cent of the sulfur given as cystine or cysteine compounds. From the increase in sulfate sulfur and cystine sulfur eliminated is calculated the oxidation of the cystine

TABLE I

	Total sulfur		Total SO ₂ -S increase over control	RSH-S as per cent of ingested cystine S ₂	Cystine sulfur.			
	Ingested	Eliminated			Intake	Output.	Eliminated.	Oxidized.
	mg.	mg	mg		mg	mg	per cent	per cent
1. Cystine 0,*	585	512	226	+6	321	248	77	91
2 postperiod days	176	189	12	+0.3		12	81	91
2. Cysteine 0,	522	438	128	+18	258	174	67	74
1 postperiod day.	88	131	39	+2		43	84	77
3. Benzyl cysteine B,	396	397	62	+52	183	157	86	40
2 postperiod days.	142	166	-11			6		
4. <i>p</i> -Cl-benzyl cysteine B,	369	356	61	+43	156	134	86	46
2 postperiod days.	142	122	-27	-2				
<i>p</i> -Cl-benzyl cysteine,	369	324	23	+52	156	91	58	24
2 postperiod days	142	137	-16	+4				
5. Diphenyluramino cysteine	585	426	98	+20	321	168	51	60
A, 1 postperiod day.	176	236	0	+19		60	69	44
6. Phenyluramino cysteine A,	585	400	36	+62	321	126	39	29
1 postperiod day.	88	104	7	+3		16	44	30
7. Phenyluramino benzyl cysteine	336	287	-30	+79	123	60	49	0
AB, 2 postperiod days.	142	114	-52	+16				
8. Diphenylacetyl cysteine A',	585	560	193	+62	321	296	92	65
2 postperiod days.	176	207	12	+6		31	102	63
9. Phenylacetyl benzyl cysteine	330	398	52	+100	117	176	150	30
A'B, 2 postperiod days	142	142		+9		0		
10. Acetylbenzyl cysteine A'B,	366	393	71	+81	153	184	120	39
2 postperiod days	142	148	-9	+22		6		
11. Diphenylhydantoin cysteine	609	473	84	+37	345	204	59	41
AC, 1 postperiod day	88	108	-14	+10		20	66	34

TABLE I—*Concluded.*

	Total sulfur.		Total SO ₂ -S increase over control.	RHS-S as per cent of ingested cystine S.	Cystine sulfur.			
	Ingested.	Eliminated			Intake.	Output.	Eliminated.	Oxidized.
	mg.	mg.	mg.		mg.	mg.	per cent	per cent
Diphenylhydantoin cystine	438	588	34	+160	172	324	188	10
AC, 2 postperiod days.	176	135	-33	+10		-31	170	0
12. Phenylhydantoinbenzyl cysteine ABC, 2 postperiod days.	336	264	-74	+88	123	40	33	0
	142	176	-20	+12		28	55	0

* The compound fed is given a bold faced number in the first column and this is also used in the text for the sake of brevity. The letters indicate the type of blocking (see p 607), 0 being used in this and Table II for no blocking and A' for ineffectual blocking. The compounds fed are in addition to a basal ration consisting of (a) 400 gm. of carrots or (b) 50 gm of bread and 75 gm of spinach. "Cystine sulfur" in the tables designates only the sulfur of the compounds added to the basal ration. The cystine sulfur periods consist of 3 days and follow 3 control days after sulfur equilibrium has been established. The figures given for the cystine periods are the sums of the 3 days and the following line the sums for the days immediately following the cystine period.

sulfur added to the basal ration, assuming that the sulfate sulfur is derived from this source.

The elimination of the ingested sulfur is seldom complete in the period in which it is administered. There is a lag of at least 1 day (sometimes 2 days) and then a return to control day levels. In nine out of fourteen cases, the extra cystine sulfur added to the basal ration is not entirely excreted in the urine by the time the control level has been resumed. Occasionally the urine of the 2nd day following the cystine period is poorer in sulfur than that of the control days (1, 4, 7, 11),¹ indicating a definite disturbance of the body processes. There is even more marked evidence of metabolic disturbance in those cases where the sulfur elimination by way of the urine exceeds the ingested sulfur as for compounds

¹ The figures set in bold face type represent the compounds listed in Table I.

9, 10, and 11. It is reasonable to infer that these β -sulfhydryl- α -amino compounds have some definite toxic properties which affect the normal sulfur metabolism. Such a conclusion is in harmony with the observations of Lewis and his coworkers (5, 6).

Introducing a radical into the amino group increases the retention of sulfur whereas if the derivative is formed by the benzyl radical at the sulfhydryl it does not consistently affect the degree of elimination of the total urinary sulfur. It seems probable that these cysteine compounds or their metabolic products are retained

TABLE II.
*Cystine Sulfur Oxidation.**

Compound No	1	2	8	5	6	11	3	4	9	10	7	12
Blocking type †	0	0	A'	A	A	AC	B	B	A/B	A/B	AB	ABC
Elimination of cystine sulfur, per cent	80	80	100	70	45	65	85	85	150	120	50	55
Oxidation of eliminated sulfur, per cent	90	80	65	45	30	30	40	45	30	40	0	0
Oxidation of sulfur fed, per cent	80	65	65	30	15	20	35	15	45	50	0	0
Oxidation, per cent	65-90			30-45			30-50			0		

Sulfur from the Basal Ration

Control days for rabbit	BLN	BO	Y	BN
Oxidation of eliminated sulfur, per cent	65	62-67	84	60-62

* A condensed table to nearest 5 multiple unit.

† See foot-note to Table I

in the animal system to be flushed out at a later time and we therefore refer to these marked plus balances as a deferred lag

The increase in the inorganic sulfate fraction of the urinary sulfur is probably a better indication of the fate of the ingested cystine sulfur than the sulfur balance itself or the increase in excreted organic sulfur. It cannot be asserted, however, that an increase in the sulfate fraction is inevitably due to the oxidation of a sulfur compound used in these experiments. There is certainly a disturbance of sulfur metabolism which suggests the

possibility that the increased sulfate sulfur as well as the sulfhydryl sulfur is derived from a stimulated metabolism of the sulfur of the active tissues (or some unsuspected passive sulfur source). Nevertheless, the increase in sulfate sulfur following the ingestion of cystine compounds is so far the most valid presumptive evidence that these foreign substances are oxidized.

No satisfactory figures are available as to the precise limits of error in physiological oxidation processes.² Many cystine feeding experiments have been reported and the oxidation as calculated ranges all the way from 40 to 100 per cent. This is nearly the range obtained from cysteine fed to a dog by Schmidt and Clark (7). Human experiments reported by Rothera (8) indicate 100 per cent oxidation, as do also subcutaneous injections into rabbits by Lewis and Root³ (5). Cystine when fed *per os* by the same investigators showed 95 per cent oxidation, with a relatively small retention. Wohlgemuth (9) who also used rabbits, reports data which indicate the same prompt elimination of cystine sulfur and the same very brief lag shown in Table I. The amount excreted was not more than 80 per cent and of this only 40 per cent was oxidized. In our own experience cystine and cysteine on passing through the rabbit body undergo oxidation amounting to from 65 to 100 per cent. All experiments reported to date are comparable and in agreement with respect to the excretion of the ingested cystine sulfur in the urine (approximately 80 per cent), but are very discordant as to the oxidation of the cystine and discrepancies can best be explained by assuming a blocking of the cystine and cysteine.

In these experiments *in vivo*, the figures for the per cent of cysteine oxidized fall well within the limits of variation in the oxidation of cystine; therefore we must conclude that the oxidation of cysteine is of the same order as cystine, as we should expect (3, 5), though the figures actually obtained for cysteine (2 *versus* 1) in Table I indicate a lesser oxidation. In any subsequent experi-

² The low order of precision is not due to inadequate analytical methods nor test management but to physiological conditions and individual variations as yet beyond control

³ However, in these last experiments there was an astonishing retention, amounting to 70 to 100 per cent, and the oxidation values may not be particularly significant

ments in the feeding of cystine or cysteine compounds, oxidation of 75 per cent or more of the cystine sulfur will be considered equally oxidizable with cystine itself; *i.e.*, as completely oxidizable under favorable conditions ⁴

The introduction of a benzyl radical into the sulfhydryl group of cysteine (3, 4) decreases the oxidation of the cystine sulfur about one-half. Hence we conclude that the sulfhydryl position is a vulnerable point in the molecule. This view is further sustained by comparing the figures obtained with phenyluramino cysteine (6) and phenyluramino benzyl cysteine (7) in which the oxidation is reduced from 30 per cent to zero by introducing the organic radical into the sulfur group. Compare also compounds (11 and 12).

After feeding diphenyluramino cystine (5) and phenyluramino cysteine (6), compounds in which the amino groups are blocked leaving exposed the other groups assumed to be vulnerable, less total sulfur was found in the urine than with cystine or cysteine, but oxidation was barely half as great, amounting to only 30 to 60 per cent instead of 75 to 100 per cent. Some protection was therefore gained by introducing the phenylsocyanate into the cystine molecule.

In 7 where a benzyl radical has been introduced into the sulfhydryl groups forming phenyluramino benzyl cysteine, simultaneously blocking both the amino and sulfhydryl groups, the sulfur elimination is not altered, but there is no oxidation of the cystine sulfur whatsoever. Similar experiments have been performed by Lewis and Root (5) with a lower recovery of total sulfur, but the oxidation is of the same order, so that the average oxidation of this compound for the four tests from the two laboratories is 29 per cent. In our previous paper (10) is reported an experiment with phenyluramino cysteine in which the substance was injected subcutaneously. The oxidation was only half that recorded in Table II. Lewis and Root made similar injections of this compound and found the oxidation nil with elimination of the cystine sulfur only half as much as when the substance was given by mouth.

⁴ The sulfur of the basal ration is oxidized to the same extent as the added cystine and cysteine (60 for the diet (a) and 85 for diet (b) as described in the foot-note to Table I)

Other derivatives structurally like phenyluramino cystine, namely diphenylacetyl cystine, phenylacetyl benzyl cysteine, and acetyl benzyl cysteine (8, 9, 10), have also been administered. Since diphenylacetyl cystine (8) is structurally much like phenyluramino cystine and phenyluramino cysteine (5, 6) similar results might have been expected from feeding it to rabbits, but it behaved more like cystine and cysteine (1, 2), the elimination of the cystine sulfur in the urine being complete and without lag and the oxidation (65 per cent) so nearly the same as for cystine itself that it can be considered of the same order. Phenylacetyl benzyl cysteine is more like phenyluramino benzyl cysteine than diphenylacetyl cystine. Its elimination is in excess of 100 per cent and the oxidation is of the same magnitude as benzyl cysteine (3, 4) (average of 3, 4, 37 per cent; average of 9, 10, 35 per cent). We now have two instances (8, 9) where the phenylacetyl radical does not function as a blocking agent. The test with phenylacetyl benzyl cysteine (9) should therefore give the same results as that with benzyl cysteine (3) and we find this to be the case. When the phenylacetyl radical is replaced by acetyl radical (10) the same degree of oxidation is obtained as with phenylacetyl derivatives.

When diphenylhydantoin cystine (11) was fed the results were strikingly like those obtained with the diphenyluramino cystine (5) for although the figures for the former are lower, the two sets are near enough alike to be considered of the same order. Somewhat less cystine sulfur was eliminated in both of these cases than in the cystine tests (1) and considerably less was oxidized.

The last test of the series was with phenylhydantoin cysteine (12) and like the corresponding cystine compound (11) this should be compared with the corresponding phenyluramino compound (7); the results of these two tests are also nearly identical. The phenylhydantoin radical is joined to the cystine and cysteine at two positions, the amino and the carboxyl. In other words, this radical blocks two of the three reactive and hence supposedly vulnerable points. The effect of this additional protection, *i.e.* at the carboxyl, is however not different from that following the introduction of the phenylisocyanate alone. Therefore this additional point covered cannot be important as a protection against oxidation of the sulfur. This view is supported by the complete protection of cysteine from oxidation when the amino and sulfhydryl are covered (7, 12), and the carboxyl is free.

In two-thirds of the cases the extra cystine sulfur added to the basal ration is not completely excreted within the cystine period inasmuch as the total sulfur eliminated is less than that added. In certain cases (9, 10, 11) there is a sporadic increase in the sulfur excretion so that the amount eliminated definitely exceeds that given as cystine sulfur. In the case of diphenylhydantoin cystine (11) there was retention in the first period, but as the experiment continued uninterruptedly into the second period, there was on the 2nd day of the period an unusual sulfur elimination. Taking these two periods together the total elimination of cystine sulfur is 100 per cent, which would suggest that the high elimination of the second period is a deferred lag, but on examination of the figures it appears that the sulfate sulfur elimination is more than 60 mg. below the control so that the high excretion is really due to a marked disturbance of the sulfur metabolism. In case of the hydantoin phenyl benzyl cysteine there was no markedly excessive elimination of total sulfur, but the sulfate sulfur fraction was even further below the control than in the test with the cystine compound. In these three cases there is a marked increase in the neutral sulfur which cannot be the deferred lag from a previous test because between two tests there was a control period in which the sulfur excretion came to the normal level⁵

It is evident from the data that aside from the elimination of the sulfur fed, there was an elimination of body sulfur, which was stimulated by these compounds. The conjugated sulfate fraction may be exceedingly important in connection with the disturbed metabolism observed, but in most cases the increase in this fraction was very small, and for each animal varied as much in the control days as during the cystine periods. Two compounds (9, 10) however, stand apart as showing a small but significantly consistent fluctuation in the ethereal sulfur, amounting to 5 mg. per day. In case of phenylacetyl benzyl cysteine the conjugated sulfur rose from 6 to 13 mg. per day at first (9) and dropped abruptly to 6 mg. in the first postperiod day, while in case of acetyl benzyl cysteine (10), it rose to 10 mg per day and immediately returned to the control value of 5 mg. when administration of this compound ceased.

⁵ For subnormal inorganic sulfate in the urine see also tests with phenyl-uramino benzyl cysteine (7).

CONCLUSIONS.

Cystine and cysteine compounds fed to rabbits when in sulfur equilibrium increase the sulfur in the urine. The evidence indicates that these compounds disturb the sulfur metabolism and that some of the excreted sulfur is derived from the body tissues.

When radicals are inhibitive to sulfur oxidation the degrees of inhibition are of similar magnitude with amino and sulfhydryl blocking, hence deamination is not necessarily an essential step in cystine catabolism.

Oxidation of cystine sulfur is reduced from one-third to one-half by introducing a benzyl radical into the sulfhydryl group; or a phenyluramino or phenylhydantoin radical into the amino group. If radicals block both of the groups, oxidation of the sulfur is completely prevented. Phenylacetyl and acetyl radicals are ineffectual for blocking and exert no influence on the oxidation of cystine sulfur but do somewhat increase the conjugated sulfate sulfur fraction of the urine.

Both phenylhydantoin cystine and cysteine depress the inorganic sulfate sulfur fraction in the urine and increase the sulfhydryl to an extent that may exceed the ingested sulfur of the experimental period.

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THE ANTIRACHITIC AND CALCIFYING PROPERTIES OF SUMMER- AND WINTER-PRODUCED DRY MILK, IRRADIATED AND NON-IRRADIATED.

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It has been known for some time that the frequency and intensity of infantile rickets is somewhat dependent upon the season of the year. According to the observations of Hess (1) and of Lasch and Miemetz (2) the spontaneous healing of rickets seems to be coincident with the increase in duration and intensity of sunshine. Hess noted that this relationship was manifested in May, whereas, Lasch and Miemetz, working in Breslau, Germany, observed its occurrence in June. In view of the abundance of data now available regarding the efficacy of ultra-violet light therapy for the treatment of rickets, these clinical observations serve primarily as confirmatory statistical evidence.

The inherent variations in the quality of the milk used in the infant dietary seem to have been given but scant consideration as a possible accessory factor contributing to the cause of the seasonal variation of rickets. In 1921, Hess and Unger (3) reported that milk from pasture-fed cows (summer-produced milk) failed to prevent the development or to decrease the severity of rickets when fed during the winter. Dry milk was used for their experiments and even though it must have been stored for several months, the investigations of Hess and Weinstock (4) and of Hart, Steenbock, and Lepkovsky (5) indicate that there would be little or no deterioration of the antirachitic factor during storage for that length of time. The failure of summer-produced milk to prevent rickets in winter is not unexpected as it is generally known that milk does not contain sufficient amount of the antirachitic factor to warrant dependence on it as a curative or prophylactic agent. The inadequacy of summer-produced milk does not, how-

ever, indicate that winter-produced milk may not be even more inferior, antirachitically considered.

In view of the benefits known to accrue as a result of direct body exposure to ultra-violet light of the sun's rays, or from artificial sources, it would seem that the milk of lactating animals might conceivably vary in antirachitic potency with variations in the degree of ultra-violet light to which the animal was exposed. Luce (6) has suggested such a relationship but particularly emphasizes the diet of the cow as an important cause of variations in the antirachitic factor. Steenbock *et al* (7) have already shown that the antirachitic properties of goat milk can be increased by exposing the animal to the rays of the quartz mercury vapor lamp. Falkenheim, Voltz, and Kirsch (8) have likewise shown that irradiation of the cow increases the antirachitic properties of the milk. These findings have a direct bearing on the question of fluctuations in the antirachitic potency of winter-produced and summer-produced milk from the northern latitudes.

Daniels, Pyle, and Brooks (9) observed that the young rats of milk-fed mothers born during February and March of 1926 were smaller, and that their rate of growth was less rapid than those born and reared under similar conditions during other months of the year. They attribute this condition to the relatively small amount of direct sunlight during the particular period in question and to substantiate this conclusion they cite the fact that pronounced improvement was noticed when irradiated milk was fed. Since numerous investigators (7, 10-16) have shown that the antirachitic properties of milk are increased by exposure to ultra-violet light, the marked difference in rate of growth of young rats as noted by Daniels, Pyle, and Brooks, when receiving irradiated and non-irradiated winter-produced milk, readily permits the inference that winter milk may be lower in antirachitic potency than summer milk, and that irradiation of the winter product provides a means of overcoming this inferiority.

EXPERIMENTAL.

Inasmuch as the antirachitic activation of milk by ultra-violet light is about to become industrialized in certain branches of the milk business, studies have been undertaken for the purpose of determining the relative antirachitic potency of milk representa-

tive of the production during typical summer and winter months; and also for the purpose of determining the effect of irradiation on such milk. For these comparative studies, dry milk,¹ desiccated by the Just double cylinder process and containing 12 per cent butter fat, was used. A quantity of the product representing winter production was obtained in March and a similar quantity was again obtained in July. Both lots of the dry milk were packed in inert gas, hermetically sealed, and stored until the following November at which time the feeding tests were started. While it has been determined that both liquid and dry milk can be effectively activated by ultra-violet light, the use of dry milk for these comparative tests was obviously more desirable. By using the desiccated product it was possible to determine the antirachitic and calcifying properties of the winter- and summer-produced milk, activated and unactivated under identical conditions and in the winter season when environmental conditions were better suited for such tests.

Portions of each of the two lots of dry milk were irradiated in thin layers of uniform thickness at a distance of 26 inches from the lamp and for the same brief period of time. A Hanovia type quartz mercury vapor lamp was used as the source of light. White rats reared under our ordinary laboratory conditions were used as the test animals. At the age of 28 to 30 days they were started on the experimental feed which consisted of the Sherman and Pappenheimer ration, No. 84, supplemented by varying quantities of the reconstituted dry milk. The animals were kept in individual cages. At the end of 48 days they were killed and autopsied; the knee-joints from two animals of each group were subsequently radiographed.² The humeri were dried, crushed, extracted, and ashed according to the method of Bethke, Steenbock, and Nelson (17) except that the extraction period was extended to 8 days to insure removal of all fat.

Table I shows the average results obtained from each group of experimental animals receiving the different milks. The feeding of graded amounts, varying between 1 and 15 cc., of non-irradiated summer-produced milk resulted in a progressive increase in ash

¹ The dry milk used was Dryco brand.

² The radiographs were prepared and interpreted by Dr. Charles Gottlieb and Dr. A. F. Hess, of New York City.

TABLE I
Comparative Antirachitic and Calcifying Properties of Summer- and Winter-Produced Milk,
Irradiated and Non-Irradiated.

Milk fed daily with Ration 84.		Final weight	Average weekly food consumption	Average ash in humeri	Rickets according to radiographs	Rickets according to costochondral junctions
Kind	Amount	gm	gm	per cent		
None	cc	42	46 0	39 25	Marked	Much enlarged.
Summer, non-irradiated	1	70	46 9	43 51	"	"
" irradiated.	1	61	44 1	48 38	Moderate	Enlarged.
" non-irradiated	4	90	52 2	48 93	Slight	Slightly enlarged.
" irradiated.	4	87	56 5	55 46	Negative	Normal.
Winter, non-irradiated	4	87	52 9	46 43	Moderate.	Enlarged.
" irradiated.	4	92	56 2	52 38	Slight.	Slightly enlarged.
Summer, non-irradiated.	9	121	59 3	55 52	"	"
" irradiated.	9	120	62 1	57 81	Negative.	Normal.
Winter, non-irradiated	9	114	55 2	50 95	Slight.	Slightly enlarged.
" irradiated.	9	120	63 4	58 27	Negative.	Normal.
Summer, non-irradiated.	15	135	57 2	60 22	"	"
" irradiated.	15	138	61 4	60 83	"	"
Winter, non-irradiated	15	145	59 1	56 08	"	"
" irradiated.	15	129	61 6	60 62	"	"

content up to 60.22 per cent. Similar amounts of non-irradiated winter-produced milk likewise caused a progressive increase in ash content of the bones, but the percentage in all cases was from 2 to 5 per cent lower than was obtained with the summer milk. The same quantities of the irradiated summer-produced milk gave results from 2 to 5 per cent higher than the same milk non-irradiated up to the 9 cc. quantity. At the 15 cc. level less than 1 per cent difference was found. In comparing the results from the irradiated and non-irradiated winter-produced milk it will be found that the irradiated product increased the ash content of the bones from $4\frac{1}{2}$ to 6 per cent more than the non-irradiated product even up to the 15 cc. level. The irradiated winter milk in all instances gave higher results than the non-irradiated summer milk; at the higher levels the irradiated winter milk gave substantially the same results as the irradiated summer milk.

The relationships shown by these data indicate a measurable difference in antirachitic and calcifying properties between summer- and winter-produced milk, but even though the winter product has less of the antirachitic factor than the summer product, its potency can be increased to a degree greater than that of the natural summer product by a brief period of irradiation with ultra-violet light. The data also indicate that under the conditions imposed by these experimental methods, the utilizable increment of antirachitic potency imparted to winter-produced milk by irradiation is greater than the utilizable increment imparted to the summer product. These results, taken as a whole, appear to confirm the existence of a significant interdependence between antirachitic properties and the degree of ultra-violet light to which the product itself, or its synthesizing agent, the body of the cow, has been exposed.

SUMMARY.

Summer-produced and winter-produced dry milk made by the Just double cylinder process tested for antirachitic and calcifying properties under identical conditions showed that the summer-produced milk possessed greater antirachitic and calcifying properties than the winter-produced milk.

Irradiation of the summer- and winter-produced dry milk imparted measurably greater antirachitic and calcifying properties

to both products. A greater increment of increase in calcifying properties was imparted to the winter-produced milk than there was to the summer-produced milk.

The results indicate that both winter- and summer-produced milk may have the same potential capacity for endowment with antirachitic and calcifying properties by ultra-violet light. The realization of this potential capacity is not attained in milk produced either in the summer or winter months. However, when the cows are subjected to the greater degree of solar irradiation prevailing in the summer months the embodiment of antirachitic and calcifying properties in the milk is greater than in the winter months when the animals are exposed to the activating rays to a much less degree. Because of the inherent conditions in the quality of the food consumed by cows in the temperate zones during the winter and summer months, the effect of such conditions on the antirachitic properties of the milk is not precluded by the data recorded herein.

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ON THE DISTRIBUTION OF THE NON-PROTEIN SULFUR OF THE BLOOD BETWEEN SERUM AND CORPUSCLES.

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The work in this paper presents the results obtained in a series of analyses made with the idea of obtaining data on the distribution of the non-protein sulfur of the blood between corpuscles and plasma.

Our determinations were made on blood in which coagulation had been prevented by the addition of 5 gm. of powdered sodium citrate per liter. We are aware of the fact that the use of an anti-coagulant of the nature of sodium citrate is undesirable in an investigation of this type; but as the conditions under which we were able to obtain our samples of human blood made the use of this substance necessary, we felt it desirable, for purposes of comparison, to collect our specimens of animal blood in the same way.

The analytical methods used were those recently described by Denis and Reed.¹ In every case the blood proteins were precipitated as soon as possible after the collection of the blood samples, although in some cases more than an hour elapsed before this procedure could be carried out due to the time required to transport the specimens from the slaughter-house or hospital.

The results obtained on the blood and plasma of the dog, beef, and goat are presented in Table I while those on human blood are given in Table II.

In the case of the three species of animals investigated the inorganic and ethereal sulfate fractions appear to be distributed

¹ Denis, W., and Reed, L., *J. Biol. Chem.*, 1926, lxxi, 191.

between corpuscles and plasma with a tendency to exist in the majority of cases in greater concentration in plasma than in whole blood. The reverse is true of the neutral sulfur which appears to be contained in largest amount in the corpuscles.

TABLE I
Non-Protein Sulfur in Dog, Beef, and Goat Whole Blood and Plasma

Animal	Mg S per 100 cc blood			Mg S per 100 cc plasma		
	Inorganic sulfate	Ethereal sulfate	Neutral sulfur	Inorganic sulfate	Ethereal sulfate	Neutral sulfur,
Dog 1.	3 64	3 14	3 30	3 60	3 30	
" 2.	3 02	2 91	5 31	5 14	4 48	0 46
" 3	3 44	0 51	7 35	3 85	1 02	2 72
" 4	2 90	0 90	6 55	3 94	0 93	1 23
" 5.	3 72	0 48	1 34	3 49	0 61	
Beef 1	3 77	0 44	1 72	4 14	0 36	0 10
" 2.	3 56	0 61	3 33	3 50	1 23	0 83
" 3	2 56	0 82	2 48	3 27	0 86	1 11
Goat 1	3 33	0 28	3 79	3 69	0 28	1 31
" 2	3 54	0 38	5 38	3 86	0 39	3 35

TABLE II
Non-Protein Sulfur in Human Blood and Plasma

Sample No	Mg S per 100 cc blood			Mg S per 100 cc plasma		
	Inorganic sulfate	Ethereal sulfate	Neutral sulfur	Inorganic sulfate	Ethereal sulfate	Neutral sulfur
1	0 55	0 07	3 31	1 10	0 51	1 83
2	0 65	0 10	3 20	0 93	0 09	2 09
3	0 49	0 14	4 32	0 99	0 48	1 74
4	0 56	0 60	3 19	1 12	0 35	2 02
5	0 45	0 15	3 24	0 85	0 91	1 72
6	0 30	0 42	3 52			2 14
7	0 28	0 34	3 73	0 50	0 96	1 87
8	0 47	0 38	4 21	0 85	0 89	1 59
9	0 34	0 96	3 49	0 66	0 66	2 54

In the nine samples of normal human blood examined the inorganic sulfate is contained almost exclusively in the plasma (if we calculate the corpuscular values as approximately 50 per cent of the volume of whole blood). The ethereal sulfates are sometimes present in larger amounts in whole blood than in plasma, although

in some cases the reverse is noted, while as in the case of the animal bloods the neutral sulfur fraction is invariably much larger in whole blood than in plasma.

Besides the difference in distribution of inorganic and ethereal

TABLE III

Average of Results on the Non-Protein Sulfur of Blood and Plasma.

Expressed as mg of S per 100 cc

		Maximum	Minimum	Average
Human whole blood.	Inorganic.	0.65	0.28	0.45
	Ethereal.	0.96	0.07	0.35
	Neutral.	5.20	3.19	3.80
Human plasma	Inorganic.	1.12	0.50	0.87
	Ethereal.	0.96	0.09	0.67
	Neutral.	2.54	1.72	1.95
Dog whole blood	Inorganic	3.72	2.90	3.35
	Ethereal	3.14	0.48	1.58
	Neutral	7.35	1.34	4.77
Dog plasma.	Inorganic.	5.14	3.49	4.01
	Ethereal	4.48	0.61	2.07
	Neutral	2.72	0.46	1.47
Beef whole blood.	Inorganic	3.77	2.56	3.29
	Ethereal.	0.82	0.44	0.62
	Neutral	3.33	1.72	2.51
Beef plasma.	Inorganic.	4.14	3.27	3.65
	Ethereal.	1.23	0.36	0.82
	Neutral.	1.11	0.10	0.68
Goat whole blood.	Inorganic.	3.54	3.33	3.43
	Ethereal.	0.38	0.28	0.33
	Neutral.	5.38	3.79	4.58
Goat plasma.	Inorganic.	3.86	3.69	3.77
	Ethereal.	0.39	0.28	0.33
	Neutral.	3.35	1.31	2.33

sulfate in animal and in human blood the level of the various sulfur fractions also presents striking differences, which are particularly noticeable in connection with the low level of inorganic sulfate in human blood with an average value of 0.45 mg. (Table III) as compared to the average figures of 3.35 for dog, 3.29 for beef, and

3.43 for goat blood. On the other hand while the average figure for neutral sulfur in human blood is somewhat lower than that found for the specimens of dog blood (3.80 mg. for human as against 4.77 for the dog) it is distinctly higher than the average values obtained for neutral sulfur on the blood of the two species of herbivorous animals analyzed.

SUMMARY.

Inorganic sulfate, ethereal sulfate, and neutral sulfur have been determined in human, dog, beef, and goat whole blood and plasma. It was found that inorganic sulfates in human blood are contained almost exclusively in the plasma, while in the case of the three species of animals examined both inorganic and ethereal sulfates are distributed between corpuscles and plasma, these fractions frequently being found in larger amounts in whole blood than in plasma.

Neutral sulfur both in human and in the animal blood was present in larger amounts in whole blood, indicating its greater concentration in the corpuscles.

ON TYROSINE AND TRYPTOPHANE DETERMINATION IN PROTEINS.

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(Received for publication, April 1, 1927.)

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I. Preliminary Observations on the Merits and Limitations of Tyrosine and Tryptophane Determinations by Means of the Phenol Reagent.

The original purpose of this investigation was to subject the Folin-Looney (1) methods for tyrosine and tryptophane determinations to a more critical study than they have yet received. A number of investigators have found those methods satisfactory, but others have condemned them on general principles, and some have been utterly unable to get any reasonable figures with them. In the meantime, the analytical figures obtained by the Folin-Looney methods have been used extensively by Cohn (2) in his calculations of the molecular weights of proteins. In these circumstances it seemed well worth while to try to clear up any uncertainties or flaws that may legitimately be ascribed to those methods. But the original purpose of an investigation may be almost lost and forgotten before a research is finished. Such has been our experience in this instance.

An elementary yet a most important question which must

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be determined in connection with any colorimetric method is that concerning the amount of the unknown substance that can be determined with a given quantity of the chromogenic reagent. This question is usually covered by studies of the range of true proportionality obtainable from different amounts of the pure substances to be determined—in this case, tyrosine and tryptophane.

In studies of the range of true proportionality obtainable in any quantitative color reaction, it is often important to try to find out the reason why the proportionality fails to hold true beyond a given point. If the failure is due merely to unequal dilution of the colored compound, then it is usually not possible to increase the range by altering the conditions. It is comparatively seldom that one encounters the dilution phenomenon as a cause of limited proportionality within the ranges measured by the ordinary colorimeters, when the standard is set at 20 mm. The two most important causes of limited proportionality are, first, that the chemical reaction between the reagent and the substance to be determined is not quite quantitative, and, second, that the reagent used by itself yields a color.

In practical colorimetry one usually has to be satisfied with a true range of proportionality between 66 per cent and 150 per cent of substance when the standard, frequently 1 mg, is taken as 100 per cent. In the methods of Folin and Looney this range of proportionality is very nearly attained when working with pure tyrosine or tryptophane. There are two reasons why the proportionality is limited to this range; the reaction between tyrosine or tryptophane and the phenol reagent is not quite complete, and the reagents alone yield an appreciable amount of blue color. It is necessary to discuss these two factors so as to make the situation clear.

The active ingredient in the phenol reagent, a phosphotungstic phosphomolybdic acid of the 1:18 series, according to Wu's nomenclature, is very unstable and quickly decomposed in alkaline solutions, and it reacts with tyrosine only in solutions sufficiently alkaline to bring about the rapid destruction of the reagent. From this combination of circumstances it follows that a relatively enormous excess of the reagent must be used in order to get complete reaction and a maximum amount of color from a given quantity

sine or tryptophane. For this reason, 0.5 mg. of tyrosine ve somewhat more than one-half as much color as is ob- from the standard, 1 mg., and 2 mg. will give a little less vice as much.

der to overcome or reduce this limitation one would natu- try to use greater amounts of the phenol reagent. But if materially larger amounts than those employed by Folin and Looney are used, precipitates and turbidities similar to those encountered in uric acid determinations are obtained, and it is often very difficult to secure perfectly clear filtrates from such mixtures. The formation of the insoluble sodium salts, represent- ing the turbidity, can fortunately be prevented in this case, just as in the case of the uric acid reagent, by the addition of lithium salts.

The phenol reagent containing the requisite amount of lithium sulfate is prepared as follows:

Transfer 100 gm of sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, and 25 gm. of sodium molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, together with 700 cc of water to a 1500 cc. Florence flask. Add 50 cc of 85 per cent phosphoric acid and 100 cc. of concentrated hydrochloric acid. Connect with a reflux condenser by means of a cork or rubber stopper wrapped in tin-foil, and boil gently for 10 hours. At the end of the boiling period add 150 gm. of lithium sulfate, 50 cc. of water, and a few drops of liquid bromine. Boil the mixture, without the condenser, for about 15 minutes to remove the excess bromine. Cool, dilute to 1 liter, and filter. The finished reagent should have no greenish tint, as this means the presence of blue reduction products which will lessen the range of true proportionality between different *small amounts* of tyro- sine or tryptophane. The reagent should be kept well protected against dust, as organic materials will gradually produce slight reductions.

By means of the phenol reagent described above we have solved the turbidity difficulty and can now use practically any desired amount of reagent. 5 cc. of this reagent seem to give the maxi- mum color obtainable, and with 5 cc. one obtains perfectly clear solutions and true proportionality in color from 1 mg. of tyrosine, he standard, and 4 mg. of tyrosine on the one hand or 0.5 mg. the other.

om the figures shown in Table I it will be seen that the color n obtainable from the phenol reagent and pure tyrosine is the most perfect reactions to be found in the field of modern etry, when the color reaction is developed under suitable

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conditions. In these determinations the standard tyrosin kept in approximately 2 N sulfuric solution, and to equal acidity in the flasks when different volumes of the standard

TABLE I
Illustrating the Proportionality of Color Obtained from Different Amounts of Tyrosine

The standard, 1 mg, was set at 20 mm

Tyrosine taken	Volume	Colorimetric reading	Tyrosine found
mg	cc	mm	mg
0.25	50	39.6	0.255
0.50	100	39.4	0.51
0.7	100	28.2	0.71
0.8	100	25.0	0.80
1.4	100	14.3	1.40
1.6	100	12.6	1.59
1.8	100	11.0	1.82
2.0	100	10.0	2.0
2.2	100	9.1	2.2
2.4	100	8.4	2.38
2.6	100	7.7	2.6
2.8	100	7.1	2.8
3.0	100	6.7	2.98
4.0	100	5.0	4.0

TABLE II
Showing That True Proportionality Is Obtained between Colors Derived from Widely Different Amounts of Tryptophane

Tryptophane found	Colorimetric reading	Tryptophane found
mg	mm	mg
0.5	39.4	0.508
0.75	26.9	0.744
1.50	13.3	1.50
2.00	10.0	2.0
3.00	6.7	2.98
4.00	5.0	4.0

taken all were diluted to 5 cc. with 2 N sulfuric acid. 40 cc water and 25 cc of sodium carbonate were then added, finally 5 cc. of the phenol reagent. 30 minutes were allowed for the development of the color.

complete
intensity

Similar experiments were made with tryptophane. Here, also, as may be seen from the figures recorded in Table II, the proportionality is perfect within the same wide range, 0.5 mg. and 4 mg.

It has already been mentioned that the reaction between the phenol reagent and tyrosine is inevitably accompanied by much destruction of the phenol reagent by the alkali. But the reaction between tyrosine and the phenol reagent is more rapid than the reaction between tryptophane and the reagent. A larger proportion of the reagent is, therefore, necessarily destroyed by the alkali and lost when one is working with tryptophane. This difference is adequately provided for in the determinations recorded in Tables I and II by the use of 5 cc. of the phenol reagent. With only 2 cc. of the reagent, and particularly when working in the presence of smaller volumes of water, the destruction of the reagent is so rapid that maximum color and a wide range of proportionality cannot be obtained with tryptophane.

Under the conditions employed by Folin and Looney, 1 mg. of tryptophane gives about 58 per cent of the color obtained from 1 mg. of tyrosine. But under the new conditions, involving the use of 5 cc. of phenol reagent, we obtain more color from tryptophane, in fact the maximum color; and we now find that 1 mg. of tryptophane gives from 88 per cent to 92 per cent of the color given by 1 mg. of tyrosine. One sample of tryptophane gave us consistently 88.5 per cent of the color obtained from tyrosine. The molecular weight of tyrosine, 181.17, is 88.7 per cent of the molecular weight of tryptophane, 204.17. The theoretical chromophoric value of tryptophane in terms of tyrosine should, therefore, be 88.7 per cent. It is clear, therefore, that we are now securing the theoretical color values within remarkably small limits of experimental error.

The finding of this value should leave no room for doubt about the fact that the chemical reaction taking place between the phenol reagent and tyrosine or tryptophane is a thoroughly sound one as a basis for quantitative measurements.

This theoretical value should also be practically useful. Tyrosine is easily obtained or can be bought, is readily purified, and in acid solutions keeps indefinitely. Tryptophane, on the other hand, is very expensive if it can be bought at all, is seldom obtained strictly pure, and standard solutions of tryptophane deteriorate

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appreciably in the course of a few weeks. By means of pure standard tyrosine solutions it is now possible to assay with accuracy the purity of a given tryptophane preparation or the tryptophane content of a given solution. Tyrosine standards in fact can now be used for the estimation of the tryptophane in the protein hydrolysates.

Our tyrosine was recrystallized as follows

Transfer 20 gm. of tyrosine to a Florence flask (capacity 2 liters). Add 200 cc. of *N* hydrochloric acid and 200 to 400 cc. of water. Heat to about 70°C., and shake until the tyrosine has dissolved. Add more water, 500 to 800 cc., and shake the solution with 2 to 3 gm. of bone-black which has been washed with dilute hydrochloric acid. Filter.

To the filtrate add the calculated quantity of sodium bicarbonate, a little at a time, with shaking. The tyrosine comes down at once. Filter on a Buchner funnel and wash with water.

The moist tyrosine is then dissolved again in a known quantity of *N* hydrochloric acid (150 cc.), and water as before. The solution this time should be water-clear. Neutralize as before and filter.

This process can be repeated if desirable without involving much loss of tyrosine, but two recrystallizations should be enough, unless the starting material is very impure.

The wide range of true proportionality between different amounts of tyrosine and tryptophane shown in Tables I and II cannot be obtained unless the reagents employed for developing the color fail to yield any perceptible color in blank, control experiments. When 5 cc. of our phenol reagent are added to a mixture of 5 cc. of 5 per cent sulfuric acid, 40 cc. of water, and 25 cc. of sodium carbonate solution, a water-clear colorless solution is obtained as soon as the reagent is destroyed by the alkali. This ideal outcome is unfortunately not attainable under the conditions prescribed by Folin and Looney. Even the very best grades of sodium cyanide which may give no trace of color with the uric acid reagent always give some color when used with the phenol reagent.

The cyanide is so handy and so useful a feature of the Folin-Looney methods that we have made many attempts to circumvent its undesirable color-producing properties, but without success. The more we have worked with sodium cyanide the more doubtful have we become as to the wisdom of retaining it in these particular methods. The trouble with the cyanide is not only

that the best grades give a color with the phenol reagent, but the poorer grades give much more, and will thus prove an incalculable source of error. The complete failure of the Folin-Looney methods in the hands of J. Warkany, for example, whose work is reported by Fürth (3), is almost certainly due to the use of an exceptionally unsuitable sample of sodium cyanide.

One particularly treacherous feature of the cyanide in this connection is that the blue color which it gives with the phenol reagent alone is no criterion as to the amount of color it will give in the presence of a mercuric salt. 4 cc. of 5 per cent sodium cyanide solution plus 1 cc. of 10 per cent mercuric sulfate solution will give several times as much color as is obtained in the absence of the mercury. Curiously enough, almost any other metallic salt, such as cadmium sulfate, and even zinc sulfate, has the same effect of greatly increasing the blue which sodium cyanide yields with the phenol reagent.

Notwithstanding these serious drawbacks to the cyanide, it is possible to use it and obtain excellent analytical results; but as it stands, the method probably can never become a standard method, because it does not adequately represent the well nigh perfect character of the underlying color reaction.

II. On the Hydrolysis of Proteins with Alkalies.

At first and in fact for a long time we were not able to secure the same tryptophane values as have been reported by Folin and Looney, except by materially altering the conditions for the precipitation of the tryptophane with mercuric sulfate. The reasons for these divergent results were finally cleared up, but only after considerable work. In the course of this study I finally decided to reexamine the hydrolysis process. For the hydrolysis of the protein for tryptophane determinations, Folin and Looney used the technique recommended by Annie Homer; namely, 48 hours boiling with 25 cc. of a 14 per cent solution of crystallized barium hydroxide per gm. of protein. While all the tryptophane is probably set free by this process it is, of course, a fact, recognized by Homer, that this relatively mild process does not produce complete hydrolysis. By adding a very little copper acetate at the end of 48 hours boiling, one obtains an intense, heat-stable, biuret reaction. One can then continue the boiling for at least 2 days

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more before the biuret reaction finally disappears. It is obvious that one cannot obtain a uniform degree of hydrolysis in 48 hours under such conditions. The result will depend on the speed of the boiling and on the amount of barium hydroxide rendered inert by absorption of carbonic acid and by combination with silicic acid from the flask. The presence of peptones is most conveniently proved by adding 5 cc. of the phenol reagent to 5 cc. of hydrolysate. There seemed to be excellent reason for using just this hydrolysis. From Homer's (4) work, and Herzfeld's (5), it would appear that barium hydroxide is the only alkali that can be used for the hydrolysis of proteins without destroying tryptophane. Both of these investigators found that sodium carbonate, even in weak concentration (0.5 per cent) produces notable destruction of tryptophane, and Herzfeld found that 9 per cent sodium hydroxide is very destructive. Furth later found that even barium hydroxide will destroy pure tryptophane, although it does not destroy it in the protein hydrolysate, while Kraus (6) claims that even in the hydrolysates there is some tryptophane destruction—with indole formation

Tryptophane is far more stable in alkaline solutions and less stable acid solutions than has heretofore been recognized. The only practical limit as to the strength of alkali which may be used for the hydrolysis of proteins for tryptophane determinations, is the loss of material through holes in the Kjeldahl flasks.

For the hydrolysis process described below one must use clean, new, Kjeldahl flasks of Pyrex glass. The only reason why Pyrex flasks are best is that they are so thick that they usually can withstand one such boiling experiment. The used flasks are perfectly good for other purposes, but cannot be used for a second prolonged digestion with sodium hydroxide. If the glass were not porous these flasks could probably be used a great many times, because the bottoms are seemingly as thick and strong as ever. But the alkali always seems to find capillary channels through which it etches invisible, but disastrous leaks.

By means of a long slender test-tube transfer into a new, clean, dry Kjeldahl flask (250 cc.) about 1 gm. of thoroughly dried protein material. The exact weight is obtained by weighing the tube before and after the transfer. Then introduce into the flask 2 cc. of butyl alcohol (to prevent foaming), a couple of short spirals made

from silver wire or silver foil (to prevent bumping), and finally 4 gm. of sodium hydroxide, in the form of 20 per cent solution. Insert into the neck of the flask a condenser made from a test-tube of such a size that it fits very loosely, yet rests firmly on the flask by means of its flange.

The mixture should be boiled for 18 to 20 hours. For this boiling it is inadvisable to apply the flame directly to the bottom of the flasks. One may succeed that way, perhaps a dozen times in succession, but it may also happen that two or three consecutive digestions are ruined because of leaks. Some form of improvised air bath should be used to secure an even application of heat. An iron crucible (diameter 7 cm.) is satisfactory. The boiling will continue perfectly smoothly, if the silver coils are right and provided that the condenser continues to function so that the butyl alcohol is not lost. It is not necessary to boil hard.

At the end of the boiling period, remove the condenser, add 10 cc. of water, and continue the boiling for 10 minutes to remove the alcohol. Then remove the flame and, from a pipette, add immediately to the hot solution, drop by drop, but rather fast, 10 cc. of 14 N sulfuric acid (200 cc. of concentrated H_2SO_4 diluted to 500 cc.). It is quite essential that the first 10 cc. of acid should be introduced into the alkaline solution while the latter is still quite hot. The addition of acid should in fact produce boiling. Unless the mixture becomes very hot the silicic acid is apt to remain in colloidal solution and the mixture will have to be discarded.

The first 10 cc. of acid are more than enough to neutralize the alkali in the flask. After the addition of 10 cc. of acid shake thoroughly and cool. Then add 5 cc. more of the 14 N acid to produce the required acidity; rinse the contents into a 100 cc. volumetric flask, dilute to volume, shake thoroughly, and filter. The filtration is slow and the funnel should be covered with a watch-glass during the 2 hour period required to get about 60 cc. of filtrate.

If more than 60 cc. of filtrate is desired, it is best to start with 2 gm. of protein material. In that case, one should add 8 gm. of sodium hydroxide and for neutralization and acidification should use 20 cc. and 10 cc. of 14 N sulfuric acid. The acidified digest is then diluted to 200 cc. before filtering.

The acidified protein hydrolysates should be kept in an ice box, or at least in the dark, unless all the desired determinations can

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be started rather promptly, for if the hydrolysates stand around exposed to light at room temperatures for many days they soon grow dark in color due to decomposition of the tryptophane. These secondary decomposition products are precipitated with the tryptophane and give a blue color with the phenol reagent, but they also impart a violet tint to the solutions so that it becomes difficult to make exact colorimetric readings.

The alkali protein hydrolysates are, of course, more or less colored without reference to the subsequent decomposition of tryptophane in the acidified filtrates. It is perhaps hazardous to try to explain the origin of these colored decomposition products except on the basis of detailed investigation, but the amount of color obtained is certainly quite unrelated to the amounts of tyrosine or of tryptophane present. Edestin, for example, yields hydrolysates which are only slightly more colored than the hydrolysates obtained from gelatin while pure egg albumin yields quite deep colored hydrolysates. To us it seems altogether probable that the discoloration obtained is due to the carbohydrate content of the different proteins

Nearly all of the color can be easily removed by shaking the acidified filtrates with a little kaolin; but as the analytical results obtained are changed little if at all by this treatment it does not seem worth while to recommend it as a regular or required procedure.

III. A New Colorimetric Method for the Estimation of Tyrosine in Protein Hydrolysates.

In the course of extended critical studies of the tryptophane precipitation by means of mercuric sulfate it soon became apparent that the empirical directions given by Folin and Looney were not entirely satisfactory. If to 8 cc. of a protein hydrolysate whose acidity is approximately normal one adds 2 cc. of a 10 per cent solution of mercuric sulfate in 2 N sulfuric acid and centrifuges at the end of 2 hours, one obtains a clear supernatant liquid. This mother liquor should contain all the tyrosine and no tryptophane. Within a few minutes after decantation this mother liquor again becomes turbid. This same phenomenon of a second precipitation is encountered whether one centrifuges and decants after 1, or 2, or several hours. At no time does one obtain a

mother liquor in which there is going on no visible further precipitation. From this phenomenon the conclusion was drawn that one step at least in the Folin-Looney process, namely the washing of tryptophane sediment with sulfuric acid and throwing away this wash liquid, could not be right. Moreover, it seemed that Folin and Looney had depended too much on the data ascertained for the separation of pure tyrosine and tryptophane. After many fruitless attempts to secure visibly sure and certain separation of tryptophane and tyrosine in protein hydrolysates we finally tried to use Millon's reaction as a test for the presence or absence of tyrosine in the tryptophane precipitates.

Tyrosine as is well known reacts only very slowly with Millon's reagent except on heating. We were, therefore, surprised to obtain immediate positive reactions for tyrosine without any heating. The tyrosine reactions obtained were like those obtained with unsubstituted phenol. A number of different experiments were then made for the purpose of discovering the cause of the prompt phenol reaction obtained with Millon's reagent, and it was soon found that prolonged contact with mercuric sulfate was the responsible factor. A preliminary short heating with mercuric sulfate was even more effective. It was also found that the original Millon's reagent was no better if as good as the sodium nitrite originally recommended by Nasse.

This discovery was extremely welcome. While extensive use has been made of the phenol reagent of Folin and Denis for the estimation of tyrosine (and tryptophane), one has always had to reckon with the fact that some careful investigators have persistently held, with Abderhalden, that the tyrosine values obtained with the phenol reagent must be very much too high, and that the only really acceptable values are those represented by the actual isolation of tyrosine. If a dependable quantitative method for tyrosine based on Millon's reaction could be found, the results obtained with protein hydrolysates ought at least to show whether the older colorimetric results have any justification. Those who have worked with gravimetric isolation methods for tyrosine have also used Millon's reaction as guide to the presence or absence of tyrosine, because this is the most specific reaction for tyrosine.

As Millon's reaction has always been used it is to be sure not perfectly selective for tyrosine because tryptophane will give a

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somewhat similar color when heated with Millon's reagent. But in the form here developed tryptophane gives no color. If present at all it will merely give a precipitate with the mercuric sulfate.

The idea of using Millon's reaction for quantitative tyrosine determinations is, of course, far from new. Several investigators have published papers on that subject. The different methods based on this reaction have given widely different results and that they could not be dependable follows from the fact that the colored compound obtained is comparatively unstable, yet has always been obtained by the aid of heat. The situation becomes entirely different, however, if the color can be developed practically instantly and at ordinary room temperature.

The development of the new method was not quite so simple a task as it seemed at first. The colored compound produced with tyrosine resembles in some of its properties the red compound obtained from Nessler's reagent and ammonia. The intensity and still more the shade of the color is determined partly by the reaction of the mixture and to a very large extent by the concentration of mercuric sulfate present. As the acidity is reduced the intensity of the color increases and the shade becomes more violet. Similar results are obtained by increasing amounts of mercuric sulfate. For accurate quantitative comparisons it is, therefore, essential that the standard and the unknown shall be substantially identical in acidity and in mercuric sulfate content. Significant inequality in these respects is revealed by the fact that the standard and the unknown are seen to have different shades when the color comparison is made. All such determinations must be discarded.

The most troublesome feature of the reaction is the tendency for the colored compound to give turbidities instead of crystal-clear solutions. It is impossible, for example, to obtain any dependable solutions in the presence of more than minute traces of tryptophane. It is, therefore, not possible to apply the reaction directly to the alkali protein hydrolysates without first removing substantially all of the tryptophane. But as this separation of tryptophane was the main object on hand the difficulty due to tryptophane was easily overcome.

The tyrosine determination on the protein hydrolysates whose preparation has been described, is as follows:

Transfer to a 15 cc. centrifuge tube 8 cc. of the protein hydrolysate and add, drop by drop, from a height of about 3 cm. 4 cc. of a 15 per cent solution of mercuric sulfate in 6 N sulfuric acid. No stirring is necessary. Let the mixture stand for 2 to 3 hours and centrifuge fairly hard for 5 minutes. Decant the supernatant liquid into a 100 cc. volumetric flask, draining thoroughly and rinsing the edge of the centrifuge tube with about 2 cc. of 0.1 N sulfuric acid. The amount of tyrosine remaining with the tryptophane is perhaps a shade more than could be accounted for on the basis of the amount of mother liquor in the tube. To the sediment in the tube add 10 cc. of a solution containing 1.5 per cent of mercuric sulfate in 2 N sulfuric acid. Stir with a fine glass rod and let stand for 10 minutes. Traces of precipitated tyrosine dissolve fairly easily in 2 N acid and the added mercuric sulfate prevents the solution of any tryptophane. At the end of 10 minutes rinse the stirring rod with 2 cc. of the same 1.5 per cent mercuric sulfate solution. Centrifuge again and transfer this wash liquid to the flask containing the original mother liquor, not omitting to rinse the edge of the centrifuge tube.

The standard is prepared as follows: Introduce into a second 100 cc. volumetric flask 5 cc. of a standard tyrosine solution in 2 N sulfuric acid containing 1 mg. of tyrosine per cc. Add 4 cc. of the 15 per cent mercuric sulfate solution and 12 cc. of the 1.5 per cent mercuric sulfate solution and about 7 cc. of 0.1 N sulfuric acid.

To the standard and the unknown must further be added 6 cc. of 7 N sulfuric acid, for the total acidity in each flask should be approximately equivalent to 100 cc. of normal acid. Heat the two flasks in boiling water for 15 minutes and then cool in cold water approximately to room temperature. Next add to each flask, with shaking, 1 cc. of 2 per cent sodium nitrite solution. Dilute to volume at once and make the color comparison without undue delay, always, of course, first reading the standard against itself so as to adjust the colorimeter, or the eye.

If the standard is set at 20 mm. then 20 divided by the reading of the unknown multiplied by 1.25 and by 5 gives the per cent of tyrosine, provided that the hydrolysate represents exactly a 1 per cent protein solution.

A few explanatory remarks concerning the tyrosine method described above may be helpful.

1. It has been made clear that there should be no undue delay in finishing the determination after the heating of the mixtures with mercuric sulfate. The reason for this limitation is the fact that after such heating with so much mercuric sulfate the protein hydrolysates always show a tendency to give another slight turbidity. A great deal of time was wasted on the tryptophane precipitation, because of the occurrence of this turbidity in the heated tyrosine-containing filtrates. As the turbidity could not be due to tyrosine it was long thought that it might represent tryptophane which had escaped the precipitation. It finally became clear, however, that the turbidity does not represent tryptophane and its only importance lies in the fact that it tends to interfere a little with the color comparison in the tyrosine determination unless that comparison is made with reasonable promptness. The turbidity is anyway very slight.

2. The reader will note that a number of solutions representing different strengths of sulfuric acid are used. Thus 14 N sulfuric acid is used for acidifying the protein hydrolysate. And we use 7 N and 0.1 N sulfuric acid, and also 6 N and 2 N sulfuric acid, for the preparation of the two mercuric sulfate solutions. No great accuracy of normality is required for these solutions and all the sulfuric acids are obtained by suitable dilutions of the 40 volume per cent or 14 N sulfuric acid employed for neutralizing and acidifying the alkaline hydrolysates.

The 6 N sulfuric acid used for the preparation of the 15 per cent mercuric sulfate solution would not have come in at all if undiluted 7 N sulfuric acid could be used, but the latter will not take up 15 per cent of mercuric sulfate. The 15 per cent mercuric sulfate solution is prepared as follows: Transfer 30 gm. of the salt to a 200 cc. volumetric flask by the help of about 80 to 90 cc. of 7 N sulfuric acid. Then add 31 cc. of water and shake or stir until complete solution is obtained. Then fill to the mark with 7 N sulfuric acid. This solution is slightly supersaturated and after a time may need to be filtered.

The weaker, 1.5 per cent, mercuric sulfate solution is made by diluting 10 cc. of the 15 per cent mercury solution plus 10 cc. of 14 N sulfuric acid with water to 100 cc.

The 0.1 N sulfuric acid, finally, is used instead of water for rinsing purposes only because water would tend to precipitate basic mercuric sulfate.

3. It will be noted that an unusually large amount of tyrosine (5 mg.) is used as the standard in this colorimetric method. Different practical reasons led to this selection. The color produced in the reaction is intense enough to permit the use of 1 mg. in 100 cc. flasks, but the brilliant red obtained is extremely difficult to read with accuracy in such dilutions. By using a 5 mg. standard one gets a color that is easy to read in the colorimeter and this standard is just about right for use with all of the tyrosine present in 8 cc. of protein hydrolysate. By using the whole of the mother liquor and washings from the tryptophane precipitation the analytical process is simplified, and, more important, the accuracy is increased.

4. The proportionality of the color derived from different amounts of tyrosine is excellent. The standard and the unknown can be at least 100 per cent apart and still yield true proportionality.

5. It may be pointed out that the new form of Millon's reaction represented in this colorimetric method is not without theoretical interest. The fact that prolonged preliminary contact, or heating, with mercuric sulfate is an essential step in the reaction clearly indicates that some organic combination between tyrosine and mercury must be an intermediary product essential to the reaction with nitrous acid. This conclusion is more or less in harmony with some recent remarks by Gibbs (7) on the same subject.

The thought will doubtless occur to some that a very convenient method for tryptophane determinations in protein hydrolysates might be obtained by determining the "total tyrosine" in the hydrolysates by means of the phenol reagent, and the true tyrosine by the process described above. The difference between the two should give the tyrosine equivalent of the tryptophane. It is unfortunately a fact, however, that tryptophane does not develop its full chromophoric power when much tyrosine is present even when a large excess of the phenol reagent is added. Accurate tryptophane results cannot, therefore, be obtained in that manner.

The suggested combination of colorimetric estimations is rather useful for another purpose, however. It supplies a fairly conclusive and reasonably accurate means of determining how much there is of other materials than tyrosine and tryptophane in protein hydrolysates which react with the phenol reagent.

IV. On Tryptophane Determinations.

The colorimetric method for the determination of tyrosine described in the preceding section is given as though it were intended solely for tyrosine determinations. It is obvious, however, that the tryptophane-mercury precipitate left in the centrifuge tubes after the removal of the tyrosine with the mother liquor and washings can be used for determination of the precipitated tryptophane. The one important question to be considered, however, is whether all the tryptophane is in this sediment. This question cannot be answered off hand in the affirmative, because small amounts of tryptophane might be present in the tyrosine mother liquor without interfering with the tyrosine determination. It is not worth while to discuss the very many experiments which have been made to find the maximum amount of tryptophane absolutely free from tyrosine

This problem was finally condensed to the following definite and simple proposition: The described tyrosine method gives all the tyrosine in the protein digest and no tryptophane value determined by means of the phenol reagent can be accepted as correct unless the mother liquor and washings also give the same, full tyrosine value.

As a matter of fact the tryptophane is precipitated as rapidly, and seems to be precipitated as completely, under the conditions selected for the given tyrosine method as under any other practical conditions. One can lengthen the time allowed for the tryptophane precipitation to 5 or 6 hours without incurring demonstrable loss of tyrosine, but by doing so one also does not get an increase of tryptophane. On the other hand, by lengthening the precipitation period to 24 hours (with casein hydrolysates) one encounters a very small loss (0.05 to 0.08 per cent) of tyrosine and the increase in the yield of tryptophane is so small (not over 0.1 per cent) that one must conclude that this extra "tryptophane" is only tyrosine.

Tryptophane Method.—The precipitation of the tryptophane is made exactly as described under the tyrosine determination.

Two different processes are available for the determination (aside from the cyanide method of Folin and Looney) and for both of these 1 mg. of tyrosine is used as the standard

First Method.—To the tryptophane sediment tube add 10 cc.

of N hydrochloric acid; stir with a fine glass rod (diameter 3 mm. or less). Heat in a beaker of boiling water for 10 minutes and then pass into this hot solution hydrogen sulfide in a slow current through a glass tube having a capillary point so long that only the capillary part can get into the liquid in the centrifuge tube. The hydrogen sulfide treatment takes only a few seconds, but must be continued till black sulfide of mercury is obtained. The fine stirring rod still in the centrifuge tube is then taken out and placed inside of the hydrogen sulfide delivery tube and both are washed with 2 cc. of 10 N sulfuric acid. The centrifuge tube is then stoppered and set aside for an hour. The 1 hour waiting period introduced at this stage is scarcely necessary, for one obtains the same tryptophane values when it is omitted. It is introduced partly as an additional precaution and partly to draw attention to the fact that tryptophane, like tyrosine, yields organic mercury compounds which are decomposed less rapidly than ordinary mercury salts.

Centrifuge and decant into a 100 cc. volumetric flask of Pyrex glass which can stand boiling. Wash off the edge of the tube with 2 cc. of 0.1 N sulfuric acid. Add 10 cc. of 0.1 N acid to the Hg_2S residue; stir, rinse the stirring rod with 2 cc. of 0.1 N acid, and centrifuge once more. Add this wash liquid to the mother liquor in the 100 cc. flask. Finally add about 15 cc. of water and boil rapidly for 4 to 5 minutes, to remove the H_2S . Cool.

Transfer 5 cc. of a standard tyrosine solution containing 1 mg. of tyrosine to another 100 cc. flask and dilute with 35 cc. of water. Add 25 cc. of saturated sodium carbonate to each flask. Dilute the contents of the boiled flask to 65 or 70 cc. Then add to each flask, with shaking, 5 cc. of the phenol reagent and let stand for 30 minutes. Dilute to volume, mix, and make the color comparison.

Calculation.—When the standard is set at 20 mm. $\frac{20}{R} \times 12.5$ gives in mg. the tyrosine equivalent of the tryptophane in the 1 gm. of protein used for the hydrolysis. This figure divided by 0.887 gives the value as tryptophane.

This colorimetric comparison is not absolutely perfect. The color given by the tryptophane is just a little less bright than that obtained from tyrosine. But the difference is so small that one

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need never be in doubt about the correct reading if one has first read the standard against itself.

The saturated carbonate solution must, of course, be free from hydrogen sulfide. As sodium carbonate rapidly takes up sulfides from rubber, the carbonate solution must not be allowed to touch rubber tubing or rubber stoppers.

The standard tyrosine solution is made by diluting 20 cc. of the strong tyrosine solution, used for tyrosine determinations, to 100 cc. with 2 N sulfuric acid.

Second Method—In this process the washed tryptophane mercury precipitate is stirred with 10 cc. of 0.1 N sulfuric acid and the stirring rod is washed with 2 cc. of 0.1 N acid. The sediment is then thrown down once more by means of the centrifuge and the wash liquid is removed by decantation. The purpose of this extra washing is to remove the excess mercuric sulfate left from the first washing. This second washing is added to the flask containing the tyrosine and a similar amount of 0.1 N sulfuric acid is then also added to the standard.

To the sediment are then added 10 cc. of N hydrochloric acid as in the first process and the stirred mixture is heated in boiling water for 30 minutes.

The sediment dissolves at once in hot hydrochloric acid, but the long heating is necessary to secure decomposition of the organic tryptophane compound

At the end of 30 minutes heating, cool, rinse into a 100 cc. volumetric flask with 30 cc. of water, and add 25 cc. of saturated sodium carbonate solution.

Prepare the standard tyrosine solution just as in the first process and then add 5 cc. of the phenol reagent to each. Let stand for 30 minutes. Then add 2 or 3 cc. of 5 per cent sodium cyanide solution to each flask, dilute to volume, and make the color comparison as before.

The sodium cyanide as here used does not affect the color, as the phenol reagent is gone. The cyanide is here added only to clear the solution from the mercury which tends to come out in the alkaline solution. If desired, 10 cc. of 10 per cent sodium sulfocyanide may be used instead of the cyanide.

Calculation.—The calculation of the tryptophane from its tyrosine equivalent is a little different in this case because of the mer-

cury. By means of many experiments with different amounts of tryptophane it was found that the liberated mercury salt diminishes the depth of color obtained from a given amount of tryptophane by 5 per cent. Instead of dividing the tyrosine equivalent of the tryptophane with the theoretical factor 0.887 the divisor must be reduced 5 per cent so that the divisor in this case is 0.843.

The two methods give substantially identical values on freshly prepared hydrolysates.

V. Tyrosine and Tryptophane in Proteins.

This is primarily a paper on the technique of tyrosine and tryptophane determinations, and practically all the check work has been done on Kahlbaum's casein ("Nach Hammarsten"). But some pure proteins, obtained from Dr. Cohn, have been analyzed by the same process.

Casein.—The vast majority of analyses on casein recorded in the literature are based on Kahlbaum's material. In this casein Folin and Looney found 1.54 per cent of tryptophane and 5.32 per cent of tyrosine. The corresponding figures now obtained are 1.4 per cent of tryptophane and 6.37 per cent of tyrosine. Hundreds of tryptophane determinations have been made on this casein and at one stage it was thought that the Folin-Looney value (1.54) was too low instead of too high. But that was because some tyrosine was constantly included in the tryptophane determinations. It is not strange that the Folin-Looney value for tryptophane should be somewhat too high since not less than 1 per cent of tyrosine remained unaccounted for in their determinations. The higher tyrosine values now found are, of course, the result of more complete hydrolysis. Casein is far more resistant to the hydrolytic action of alkalis than are the other proteins examined and it is only here that the new methods give so much higher tyrosine values.

A highly purified casein obtained from Dr. Cohn was also analyzed. The filtered hydrolysate obtained from this casein had no more color than the very slight straw-yellow which one obtains in gelatin hydrolysates. The other casein hydrolysates are light brown. This difference indicates that Kahlbaum's casein probably contains some milk sugar. The tryptophane precipitate from Cohn's casein has almost the pure straw color

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that is obtained from colorless tryptophane solutions. Cohn's casein contained 1.4 per cent of tryptophane and 6.55 per cent of tyrosine.

Egg Albumin.—In ovalbumin Folin and Looney found 4.2 per cent of tyrosine and 1.23 per cent of tryptophane. Here the barium hydroxide hydrolysis has evidently set free all the tyrosine as well as the tryptophane. Here also the lower tyrosine content reduces the danger of getting tyrosine in the tryptophane precipitate; but, on the other hand, the tryptophane precipitation is not quite complete. The figures now obtained for ovalbumin are 1.3 per cent tryptophane and 4.0 per cent tyrosine. It may be remarked in passing that even before the introduction of the hydrolysis with sodium hydroxide or the new tyrosine method, Dr. Ciocalteu had obtained 1.33 per cent tryptophane and 4.0 per cent tyrosine in ovalbumin. At that time the hydrolysates were made 2 N in acidity and the tryptophane was precipitated in 24 hours after the addition of 2 cc. of 2 N sulfuric acid containing 10 per cent of mercuric sulfate.

In the case of egg albumin it is advisable to decolorize with about 1 gm. of kaolin. The figures recorded above were obtained on such decolorized filtrates.

Edestin.—According to Folin and Looney, edestin should contain 1.40 per cent tryptophane and 5.7 per cent tyrosine. The latter figure must be due to some serious error, for the Folin-Looney process could not possibly give such a tyrosine figure for edestin. In his recent paper on the subject, Looney records the tyrosine of edestin as 4.58 per cent and the tryptophane as 1.52 per cent. These figures are substantially identical with the values obtained by the methods described in this paper—tryptophane 1.51 per cent, tyrosine 4.53 per cent.

The second decimals in these values are not, of course, to be insisted upon. Edestin contains 1.5 per cent of tryptophane and 4.5 per cent of tyrosine.

Gliadin.—In gliadin Folin and Looney found 1.14 per cent of tryptophane and 3.4 per cent of tyrosine. These figures were later revised by Looney to 1.1 per cent of tryptophane and 3.04 per cent of tyrosine. The corresponding values now obtained are 0.84 per cent tryptophane and 3.1 per cent of tyrosine.

Looney's figures represent the average of several determinations

and we have made a considerable number of determinations, using in part the same samples of gliadin as had been used by Looney. While his and our tyrosine values agree very well there is a quite material difference in our respective tryptophane figures. It is perhaps best not to try to explain these differences at present. If we were dealing with analyses of a less experienced investigator in this field than Looney, it might be permissible to assume that here was a case where the cyanide-mercury combination of the Folin-Looney process had conspired to introduce an excessive deviation from true proportionality in the color comparison. If our figure is correct there would be only about 0.7 mg. of tryptophane in the 8 cc. of hydrolysate used, and this would be compared against the 1 mg. tryptophane standard. Any error that could creep in would, therefore, certainly be on the plus side. It seems scarcely possible, however, that an error of the magnitude here involved could be due only to a fluctuating cyanide-mercury blank. But it is particularly in cases of this kind where the tryptophane content is low, that we should expect the process described in this paper to yield more dependable figures.

Zein.—The tyrosine determinations were made in the usual manner on 8 cc. of hydrolysate from which the tryptophane had been removed by a 2 hour precipitation with mercuric sulfate. For the tryptophane determinations 25 cc. of hydrolysate plus 12 cc. of 15 per cent mercuric sulfate were used (in 50 cc. centrifuge tubes). The standard contained only 0.4 mg. of tyrosine. The zein used in these analyses came originally from Osborne's laboratory. It contains 0.17 per cent of tryptophane and 5.9 per cent of tyrosine.

Folin and Looney referred to the tryptophane content of zein, as of gelatin, only in tabular form and gave it as 0, instead of a trace, as they well might have done purely on the basis of the fact that a turbidity is obtained on the addition of mercuric sulfate to the hydrolysates. At that time such small traces seemed unimportant. Now the situation is different. The smaller the quantity of a given amino acid the greater is its significance for molecular weight calculations, provided that the analyzed material is pure.

Incidentally it may be mentioned that a precipitation method which is capable of yielding 0.17 per cent of tryptophane from 1

per cent zein hydrolysates should be very dependable when applied to corresponding hydrolysates from other proteins which contain from 5 to 8 times as much tryptophane as does zein. In this case, $2\frac{1}{2}$ hours were allowed for the tryptophane precipitation and the precipitate was washed as usual with 2 N acid containing 1.5 per cent of mercuric sulfate. The tryptophane figure, 0.17 per cent, should probably be regarded as a minimum value, since one cannot ascribe absolute insolubility to the tryptophane-mercury precipitate.

VI. The Preparation of Pure Mercuric Sulfate.

In the preceding pages we have pointed out that different samples of sodium cyanide give different amounts of color with the phenol reagent, and that the color thus obtained is greatly intensified in the presence of mercuric sulfate. We have also found that different samples of mercuric sulfate give different amounts of color with a given solution of sodium cyanide. The reason for these differences is evidently the fact that practically all brands of mercuric sulfate contain more or less of mercurous sulfate, and may contain other reducing salts such as ferrous sulfate.

We have examined a large number of mercuric sulfate samples, including one French and one German preparation. The sample manufactured by Baker and Adamson was different from all the others, and the only one which was substantially pure. It contained only traces of iron and of mercurous sulfate.

Since it is important that only pure mercuric sulfate should be used for the quantitative estimation of tryptophane, we set ourselves the task of working out a suitable method for recrystallizing it. We have found no useful information, bearing on this point, in the literature. The method described below is based on the discovery that mercuric sulfate, in the presence of the right amount of sulfuric acid, is extremely soluble in water, up to more than 50 per cent. From such concentrated solutions it is almost quantitatively precipitated, in snow-white crystalline condition, by the addition of more sulfuric acid. The process given below is based on the use of 1 kilo of the crude, bought material; but the proportions given, 1 cc. of concentrated sulfuric acid to 6 gm. of the salt, will work equally well with smaller amounts.

Transfer to a 4 liter Pyrex beaker 1 kilo of mercuric sulfate, and 150 cc. of concentrated sulfuric acid. Stir until a uniform paste is obtained. Then add water slowly with constant stirring (heavy glass rod or porcelain spoon) until none of the original heavy salt remains at the bottom of the beaker. More or less black dirt and mercurous sulfate will remain suspended in the solution. 1700 to 1800 cc. of water will be required to dissolve the mercuric sulfate. Filter through a good quality heavy filter paper into a 4 liter flask. This filtration is slow; the first part of the filtrate may be a little turbid and should be poured back on the funnel. The color of the filtrate is largely determined by the amount of iron present in the original salt.

To the filtrate in the flask add gradually, with shaking, 450 cc. of concentrated sulfuric acid. Considerable heat is, of course, generated during the addition of the acid, and we prefer to keep the flask in a large kettle of cold water during the process. The snow-white precipitate comes out at once, and settles rapidly. Remove by decantation about 100 cc. of the supernatant liquid and to it add 10 cc. of concentrated sulfuric acid. If more than a very slight precipitate is obtained, add more sulfuric acid to the original mixture. 450 cc. should be enough, however. Cool, and filter on a Buchner funnel through a hardened filter paper. Wash with 400 cc. of 25 volume per cent of sulfuric acid. Drain thoroughly by suction with a water pump.

Blow the precipitate back into a dry 4 liter beaker and remove the filter paper. Add 150 cc. of concentrated pure sulfuric acid, stir to a paste, and dissolve, as before, by the gradual addition of water.

Filtration at this stage is superfluous, but should not be omitted unless the solution is *really* clear. Repeat the precipitation by the addition of sulfuric acid just as was done with the first filtrate. Cool, filter, and wash with cold dilute sulfuric acid (1 volume of acid to 3 volumes of water).

Blow the precipitate into a *dry* beaker, pick out the hardened filter paper, and add to the precipitate, gradually and with stirring, 500 cc. of an equal mixture of alcohol and ether. Stir very thoroughly and filter the mixture on a Buchner funnel. In this final filtration it is not necessary to use a hardened filter. A little additional alcohol-ether mixture may be used for rinsing the last of the precipitate in the beaker onto the funnel, but none should be used for washing the precipitate. It takes very little water or moisture to give insoluble yellow basic sulfate, when practically all the sulfuric acid has been thus removed, but if the directions are followed no basic sulfate is obtained at any stage.

Transfer the precipitate to a new, porous, clay plate. Place the plate on a double layer of filter paper, away from too much atmospheric moisture, and allow to dry, at room temperature. Yield 750 gm.

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TITRATION CURVES OF TAURINE AND OF CYSTEIC ACID.

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Taurine and cysteic acid are of interest both from a physiological and chemical view-point. They are concerned in the synthesis of the sulfur-containing bile acids. Investigations which have been carried out in this laboratory show that taurine, when ingested, is excreted in the urine unchanged, while cysteic acid is deaminized, but the sulfur-containing radical likewise undergoes no change in the animal organism (1). Chemically taurine and cysteic acid behave like the ordinary amino acids. Both substances liberate their nitrogen in 4 minutes when treated with nitrous acid.

Taurine and cysteic acid differ from the ordinary amino acids in that they contain a sulfonic acid group. Since it is usually assumed that sulfonic acid compounds are more highly dissociated than the corresponding carboxylic acids, it appeared of interest to determine, if possible, the dissociation constants of taurine and of cysteic acid. The titration curves were carried out in the usual manner by adding varying quantities of standard acid or alkali to the solution of ampholyte. Estimation of the pH was made at 25°C. in the usual manner with the aid of the Clark cell, 0.1 N KCl-calomel electrode, saturated KCl bridge, and a Leeds and Northrup hydrogen ion potentiometer. The electrometric readings were interpreted with the assistance of the tables of Schmidt and Hoagland (2).

Taurine was obtained from the abalone, *Haliotis*, according to the method which has been described by Schmidt and Watson (3). Cysteic acid was prepared from cystine according to the method described by Friedmann (4). It was purified by recrystallization of the copper salt. The two crystal forms de-

scribed by Friedmann were obtained. They gave identical titration curves.

The results are graphically plotted in Fig. 1. In order to determine the dissociation constants it is necessary to convert the titration curves into dissociation curves according to the method described by Michaelis (5) and by Clark (6). For taurine K_a has the value of 1.8×10^{-9} . Winkelblech (7) previously reported 1.6×10^{-9} . The value of K_{a_2} for cysteic acid is 2×10^{-9} .

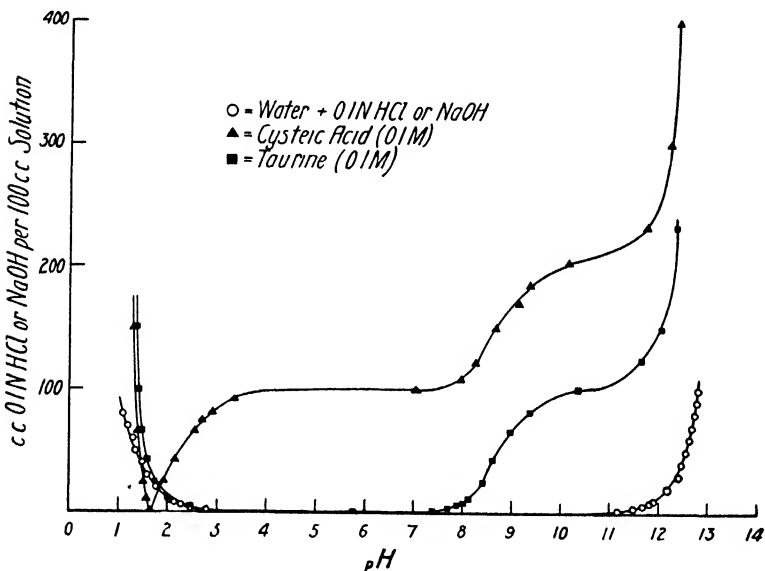


FIG. 1.

In attempting to calculate the value for K_b of taurine and of cysteic acid and K_{a_1} of cysteic acid, certain difficulties are encountered. These are due to the presence of the strong sulfonic acid group in the molecule. Below pH 2 the accuracy in the estimation of acidity decreases very rapidly. Moreover, in the region of high acidity we are confronted with the so called anomalies of strong electrolytes. It is, however, possible to determine approximately the magnitude of these constants in the following manner. From the taurine curve the isoelectric point may be determined by taking the midpoint between the points on the acid and alkali

dissociation curves where the concentrations of free acid and salt are equal. This gives a value of 5.1. The basic dissociation constant was calculated from the equation

$$I = \left(K_w \frac{K_a}{K_b} \right)^{\frac{1}{2}}$$

Its value is 3×10^{-13} . This figure is not absolute but represents rather an approximation. Winkelblech (7) recognized that the amino group in taurine is only very slightly dissociated when he stated, "Eine ganz geringfügige Salzbildung scheint vorhanden zu sein. Allerdings ist kein Chlorhydrid beim Taurin bekannt."

Although the isoelectric point is not necessarily identical with the pH of the dissolved substance, no great error will probably result if in the case of cysteic acid we assume that they are identical. We shall take the value of 1.6 for the isoelectric point of cysteic acid. The magnitude of K_b will not differ greatly from the value found for taurine. It probably will be slightly less. We shall assume $K_b = 2 \times 10^{-13}$. Using the method suggested by Levene and Simms (8) the calculated value for K_{a_1} is 1.3×10^{-2} . This value is an approximation. It indicates, however, that the primary acid group in cysteic acid is more highly dissociated than the primary carboxylic acid group in glutamic or aspartic acid.

SUMMARY.

Titration curves of taurine and of cysteic acid have been experimentally obtained. From these it is found that K_a (taurine) = 1.8×10^{-9} and K_{a_2} (cysteic acid) = 2×10^{-9} . By making certain assumptions the following values were obtained by calculation: K_b (taurine) = 3×10^{-13} , K_{a_1} (cysteic acid) = 1.3×10^{-2} . It was assumed that K_b (cysteic acid) = 2×10^{-13} .

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RICKETS IN RATS.

I. METABOLISM STUDIES ON HIGH CALCIUM-LOW PHOSPHORUS DIETS.

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To produce rickets in rats, the diet must be deficient in two essentials: (1) absence of vitamin D, (2) alteration in the salt content. These conclusions, according to Park (1), represent the present accepted theory of the etiology of rickets. The knowledge of metabolism in rickets of rats is quite recent (2, 3).

To investigate the calcium and phosphorus metabolism of rats from normal through the development of rickets was our purpose. As confirming evidence of rickets we have studied the blood and bones. We present data on (1) blood serum analyses, (2) histologic examination of the bones, (3) bone analyses, (4) metabolism of calcium and phosphorus.

Conduct of Experiment.

The study was conducted in March, April, and May, 1925. Young white rats of pure albino stock from the Albino Supply Company, Inc., Philadelphia, were kept with their mothers until weaned. The mothers and young had access to our stock diet (Sherman Diet B) (4) consisting of 33.3 per cent of whole dried milk, 65.4 per cent whole wheat, 1.3 per cent NaCl. Rats 28 to 30 days old and weighing approximately 45 gm. were used.

The subsequent experimental diet was of the high calcium-low phosphorus type and was deficient in vitamin D. The food was made from grains of seed quality, carefully weighed and ground and kept in tight containers. Diet 3143 of McCollum (5) and a modification of this diet (No. 2965) recently described by Steenbock and Black (6) were used. The latter diet is composed of yel-

low corn 76 parts, wheat gluten 20, calcium carbonate 3, and sodium chloride 1. 10 gm. of lard were added to each 100 gm. of diet in order to give the food a more pasty consistency and to prevent scattering. The two diets yielded essentially the same results for blood and bone analyses and as only rats on the latter diet were used for the metabolism experiments, the discussion will be limited to rats on the modified Steenbock diet. By analysis it contained 1.08 per cent calcium and 0.254 per cent phosphorus which is in the ratio of $\text{Ca:P} = 4.25$. The animals were fed this diet and distilled water for 5 weeks.

The rats were placed in two types of cages, the Hopkins cage, slightly modified by Dr. James Gamble, and the stock cage. The animals in the stock cages furnished data for the blood serum analyses, bone histology, and bone analyses, and were killed at weekly intervals. Those in the Hopkins cages furnished similar data and in addition data for metabolic studies. They were killed at the end of the experiment. All were killed by bleeding from the carotid artery after primary ether anesthesia and the blood of two to four rats was pooled for analysis. The animals were autopsied the same day, weighed, and measured. The bones were removed for histological examination and for analysis of calcium and phosphorus.

Methods.

The blood of two to four rats was received into clean Pyrex centrifuge tubes and the serum separated at once. The serum was analyzed within a half hour for inorganic phosphorus by the Briggs modification of the Bell-Doisy colorimetric method (7). The calcium was precipitated directly as oxalate and titrated with 0.01 N KMnO_4 according to the Kramer-Tisdall method (8). The bones for section were decalcified in Müller's fluid and then sectioned and stained with hematoxylin and eosin. A sample of the food used was ashed in platinum at low heat in an electric muffle furnace. The ash was dissolved in HCl and made to a known volume. Aliquots were taken for analysis and were made neutral to methyl red with ammonia. Calcium was precipitated as oxalate and titrated with permanganate. Phosphorus was precipitated as molybdate; the yellow precipitate was dissolved and precipitated as NH_4MgPO_4 , which was converted to $\text{Mg}_2\text{P}_2\text{O}_7$ and weighed. The bones were ashed with HNO_3 and the Ca and P determined as in food. The urine was made to known volume and an aliquot taken for analysis. The stools were dried three times with alcohol on the water bath, ground to a fine powder, and an aliquot taken for analysis. Both urine and stool were ashed with H_2SO_4 and HNO_3 and the calcium and phosphorus determined as in foods.

Progress of Experiment.

The young rats on the modified Steenbock diet behaved normally. They became accustomed to the diet quickly. They did not gain regularly nor were their gains uniform. For the last 2 weeks they lost weight.

Enlargement of the tibiae of the rats on the experimental diet was observed after 7 to 10 days and was marked at 3 weeks. The chests were more easily compressible as rickets developed. At the end of the experiment the animals on the low phosphorus diet had poor pelts. They were also weak.

TABLE I
Blood Serum on Rickets-Producing Diet.

Days on experiment	Serum			
	Ca		P	
	Experiment I	Experiment II	Experiment I	Experiment II
	mg per cent	mg per cent	mg per cent	mg per cent
0*	10.2		9.8	
7	10.9	11.0	5.4	4.8
14	9.6	12.0	4.5	4.0
21	9.2	11.0	3.9	2.4
24	11.2	9.8	3.5	4.3
28	11.0	13.0	5.9	4.2
31	10.7	12.2	3.7	3.3
35	10.4	10.8	2.9	4.3

* Normal at 30 days.

Results.

1. *Blood Serum Analyses.*—The blood findings are those typical of rickets produced on this diet—normal or slightly elevated calcium and low phosphorus of the serum. The results are given in Table I. These values have been repeated many times in our laboratory and two sets of experiments in close agreement are shown. Beginning with normal values of 10 mg. of Ca and 10 mg. of P per 100 cc. of serum, the P drops to 3 to 4 mg. at the 3rd week and remains at this level. The Ca remains normal or slightly elevated.

2. *Histologic Examination of the Bones.*—The histologic evidence bears out the blood findings. As is customary on the Steenbock diet, our slightly modified Steenbock diet produced uniform and severe rickets. There was slight, if any, evidence of fresh or recent lime salt deposition.

3. *Bone Analyses*.—The data consist of calcium determinations on the air-dried bones which had been separated from the soft parts by boiling. They show that there is less calcium in the bones of the ricketic animals than in normals of the same age. When the calcium in the bones is calculated on a percentage basis, Hammett's (9) data show that the normal dry femur at 30 days contains 15.9 per cent Ca and at 65 days 21.1 per cent Ca. Our data show 14.0 per cent Ca after 1 week and 15.0 per cent after 4 weeks on the modified Steenbock diet. From Hammett's data we calculate that the calcium gain corresponds to a positive balance of one-third of the normal value. Therefore during the development of rickets, calcium deposition has not ceased, but is proceeding at only one-third the normal rate.

4. *Metabolism of Calcium and Phosphorus*.—Data were obtained only from those animals in the Hopkins metabolism cages. This cage is so arranged as to permit accurate estimation of the weight of food consumed and complete separation of urine and feces. The cage rests in a large glass funnel. The funnels were rinsed with hot water daily and the cages cleaned thoroughly once a week. The washings were filtered and the filtrate added to the urine. The residue was dried and added to the food spilled, which had been picked out from the dry feces and weighed. Urine and feces were collected in weekly lots and combined for each group of six rats. No known losses of excreta occurred. Known calcium solutions which trickled through the cage, drop by drop, were recovered quantitatively.

Six rats were placed in pairs in three metabolism cages. Animals in these cages showed smaller gains in weight than those in the stock cages. The average initial weight of 47.5 gm. increased to 68 gm. after 21 days on the modified Steenbock diet. Our rats grew at only one-half the normal rate, which is 14 gm. per rat per week, for the first 3 weeks. Continuing on the same diet for 14 days longer, they lost weight and averaged only 63 gm.

The total period was 34 days, but the data are calculated to the basis of 35 days, five weekly periods. The total food spilled by scattering amounts to 1 per cent of the food offered. The total daily net food intake per rat is 4.4 gm. or 30.8 gm. per week, equivalent to 119 calories per week. The animals upon the same diet in larger cages showed daily food intakes per rat greater by

0.5 gm. The feces were dark, well formed, and were never diarrheal. The dried stool was quite constant in weight and averaged 3.6 gm. per rat per week.

Discussion of Previous Diet.—The diet of the mothers and of the young rats prior to the experiment is an important factor in the production of rickets, as has recently been demonstrated by Korenchevsky (10) and Smith and Chick (11). The effect of the maintenance diet itself has been amply studied by Sherman (1924) and is adequate for the demands of nutrition and reproduction. The calcium phosphorus ratio of this diet, by calculation from Sherman's analysis, is 0.65 and is therefore a high phosphorus diet. Analyses of its bases and acids show an excess of 10 cc of 0.1 N in acid per 100 gm. of diet and so it is practically neutral.

Experimental Diet.

Salt Content.—Experimental diet of high calcium and low phosphorus content was selected because in our experience it regularly produced constant and severe rickets. The diet of the Lister Institute (10, 11), although it may more closely approximate the salt ration in human dietaries, does not seem to produce a rickets either so constant or severe.

Fat Content.—10 per cent of lard was added to the diet, so that it is not the same as the Steenbock diet. Although our primary consideration was modification of the physical characteristics other changes were also thereby introduced. The diet became more concentrated so that its caloric value altered from 3.6 to 4.1 calories per gm., a 14 per cent increase. Second, the other constituents were therefore eaten in less amount. Third, the relationship between the fat and mineral salts may alter the requirements of the latter. Rübner has suggested such a relationship between fat and calcium in the diets of the Japanese. This does not however affect our experiment because, on the modified diet, rickets of approximately the same degree as on the original diet is produced.

Results.

The values for calcium and phosphorus of the food, urine, and feces, and the calcium and phosphorus balances are given in Table

II. They represent weekly periods, except that the food was measured for the whole period of 5 weeks (34 days).

Paths of Excretion.—The calcium in the urine averages 23.0 per cent of the total calcium excretion or 17.5 of the intake. The calcium in the stool averages 77 per cent of the total calcium excretion or 58 per cent of the intake. The phosphorus in the urine represents 5 per cent of the phosphorus excretion or 4 per cent of

TABLE II
Metabolism of Six Rats for Calcium and Phosphorus on Modified Steenbock Diet.

Figures in terms of one rat per week.

Period	Calcium						Phosphorus					
	Intake	Urine	Stool	Total output.	Balance		Intake	Urine	Stool	Total output	Balance	
wks.	mg.	mg.	mg.	mg.	mg.	per cent of intake	mg.	mg.	mg.	mg.	mg.	per cent of intake
1		32	210	242	(+91)*			2	55	57	(+20)*	
2		43	184	227	(+106)*			2	55	57	(+20)*	
3		99	238	337	(-6)*			4	55	59	(+18)*	
4		33	191	224	(+109)*			2	50	52	(+25)*	
5		85	142	227	(+106)*			4	50	54	(+23)*	
Total for five periods	1667	292	965	1257	+410		387	14	265	279	+106	
Average per wk.	333	58	193	251	82	25	77	3	53	56	+21	27

* The values in parentheses represent the calculated balance when the food is allotted in equal amounts for each week.

the intake. The phosphorus in the stool amounts to 95 per cent of the excretion or 69 per cent of the intake.

From a consideration of the data of Boas (3, 12), Telfer (13, 14), and Orr *et al.* (15), it is highly probable that the paths of excretion are the same in the normal and ricketic subject. Alterations in the excretion in the urine and stool would therefore depend primarily upon the composition of the diet, especially with regard to its salt content, and not upon the defect of metabolism present in rickets.

Balance of Calcium.

The balances of calcium are positive and average 82 mg. of calcium per week or 25 per cent of the intake during the period when rickets developed, as is shown in Table II. The full effects of the diet upon the production of rickets are not present during the 1st week, as indicated by the decreasing balance when the food is arbitrarily allotted in equal amounts for each week. The period during which active rickets is developing shows a marked diminution of the calcium balance, but not a negative balance. The retention is far below normal as can readily be computed from the analyses of Sherman (16) which are substantiated by Medes (17). The normal total calcium of the rat at 30 days is 311 mg. and at 60 days 1023 mg. This represents a retention of 166 mg. per week. During the development of rickets 50 per cent of this amount was retained. These findings are corroborated by Boas (12) and Medes (17).

Balance of Phosphorus.

The balances of phosphorus, given in Table II, are also positive and average 21 mg. of phosphorus per week or 27 per cent of the intake during the period on the rickets-producing diet. These balances are practically constant if one assumes equal weekly food intakes. The comparison of these values with the normal phosphorus retention is also simple. Due to the recent paper of Sherman (18), the total amount of phosphorus read from smoothed curves of the average for males and females at 28 days is 0.26 gm. and at 61 days is 0.76 gm. or 105 mg. retention per week. Medes' (17) values calculated on the same basis equal 133 mg. Our values amount to 20 per cent of the normal retention as established by Sherman. Boas (3, 12) and Medes (17) corroborate our findings.

Ratio of Calcium and Phosphorus Retention.

The normal ratio of Ca:P retained is easily calculated. If the normal calcium balance is 166 mg. and the normal phosphorus balance 105 mg., the Ca:P ratio is 1.58. This value is in agreement with that of Boas (12) of Ca:P = 1.65, and in her later communication (3) of Ca:P = 1.58 for rats receiving cod liver oil.

The ratio of the retention of Ca:P during the period when our

experimental animals were on the rickets-producing diet is 82:21 or 3.9. Medes (17) and Orr and collaborators (15) show similar high ratios. Hence our data in agreement with theirs indicate that on a high calcium-low phosphorus diet a large deficit occurs in the phosphorus retention not only absolutely but also relative to the calcium retention.

DISCUSSION.

In the metabolism of rickets in rats on a diet deficient in vitamin D, and relatively high in calcium and low in phosphorus the significance of the ratio of Ca:P has been considered. The absolute amount of calcium and phosphorus, food intakes and the resultant rate of growth, other constituents of the diet, acid-base value of the diet, its content of other salts, and distribution of the retained materials, are also factors to be considered. It is apparent that rickets may result from widely different types of diet. A comparison of our data with that of Boas, shows a number of similarities in the metabolism of rickets although the diets were quite different. Both showed positive, but diminished balances of calcium and phosphorus. Ours have the same deviations from the normal with respect to calcium and greater deficits in respect to phosphorus. This has been interpreted as resulting from the relative phosphorus deficit in the diet. The paths of excretion show different percentages excreted in the feces and urine, contrasting, as was to be anticipated, with those in Boas' study of the metabolism of rickets, but quite similar to those of Orr, Holt, Wilkins, and Boone in their study of high calcium-low phosphorus diets in ricketic children.

The retention of calcium and phosphorus given in this paper represents the gross or total retention. If one organ gains at the expense of another, there is besides the gross metabolism, an internal or intermediary metabolism. Data in this field should advance our knowledge of mineral metabolism of rickets.

CONCLUSION.

On a diet high in calcium and low in phosphorus and deficient in vitamin D, by which rickets is produced:

1. The calcium and phosphorus balances were both positive.
2. The calcium retention was 50 per cent of the normal and the

phosphorus was but 20 per cent of the normal—thus resulting in an excess proportion of calcium to phosphorus retained.

We wish to express our thanks to Dr. E. A. Park for his examination of the bone sections, and Miss D. Jackson for the technical work involved in their preparation, and to Miss C. Rothwell and Miss H. Bennett, who made the chemical determinations on the blood serum, bones, food, urine, and feces.

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RICKETS IN RATS.

II. THE EFFECT OF PHOSPHATE ADDED TO THE DIET OF RICKETIC RATS.

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In ricketic animals lime salt deposition may be brought about in several ways. by feeding vitamin D or irradiated food, by radiation, by starvation, or by change toward normal in the salt composition of the diet. In a ricketic child the equilibrium between calcium and phosphorus in the blood serum is unstable. An amount of phosphorus added to the diet which causes no change in a normal child, may in a ricketic child lower the calcium markedly, even to produce tetany¹. This clinical observation suggested the present study. It was hoped that the effect of salt additions could be convincingly demonstrated by metabolism studies upon experimental animals.

Rats previously made ricketic on high calcium-low phosphorus diets were given extra phosphate, so that the phosphorus was equal to the calcium by weight. No change was made in the light or vitamin factors.

The animals were studied as to the following points: (1) blood serum analyses, (2) histologic examination of the bones, (3) bone analyses, and (4) metabolism of calcium and phosphorus.

This is an extension of the previous experiment (1), with a different group of rats, and was conducted with the same technique by the same personnel at the same time.

Young white albino rats at 28 to 30 days and weighing 45 gm. were used. They had been kept with their mothers on the Sherman Diet B, 33.3 per cent whole dried milk, 65.4 per cent whole

¹ This observation was made in a personal communication by Dr. James Gamble, and confirmed in this department by Dr. E. A. Park

wheat, and 1.3 per cent NaCl, until weaned. They were then placed on the Steenbock diet (2) to which 10 per cent lard had been added. By analysis this contained 1.08 per cent of Ca and 0.254 per cent of P which is in the ratio of $\text{Ca:P} = 4.25$. They were kept upon this diet for 21 days. At the end of this period the rats received added phosphorus in the form of NaH_2PO_4 . When 4.24 gm. of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were added per 100 gm. of diet, by analysis, it contained 1.06 per cent of Ca and 1.121 per cent of P which is in the ratio of $\text{Ca:P} = 0.95$. The Ca:P ratio of approximately 1 was chosen to avoid a high P-low Ca rickets and



FIG 1 Effect of added phosphate upon a ricketic rat. The rat shows carpopedal spasm of the hind leg.

was selected as optimal for maximum calcium deposition. This alteration in the salt content is an empirical value given by McCollum (3) for this particular diet.

Progress of Experiment.

The young rats on the modified Steenbock diet behaved quite uniformly. When phosphate was added to the diet the animals did not eat normally for several days and then gradually took the usual amount of food. They were very quiet for the first few days after the addition of phosphate, but later seemed much more active than those on the control diet. Three rats after 1 week of added phosphate developed intermittent spasms of the hind limb

with spasticity and inversion of hind feet. A photograph is shown in Fig. 1. They were hyperexcitable and appeared rather weak as compared with their ricketic controls. A rat in the stock cage died in convulsions; another developed an ophthalmia. As a result of the phosphate addition the feces became white. For the 1st week the feces were small in amount. The animals lost weight (due to inadequate food intake) during the 1st week after the addition of phosphate, but during the 2nd week they were again gaining, while the controls were losing weight. The initial weight averaged 43 gm. After 21 days on the rickets-producing diet the rats averaged 63 gm. When phosphate was added they lost weight. After 7 days they weighed 56 gm. and gained to 60 gm. after 14 days.

TABLE I.

Value of the Blood Serum of Rats Given the Rickets-Producing Diet and Added Phosphate. Experimental or Added Phosphate Group.

Days of experiment	Days on added PO_4	Serum			
		Ca		P	
		Experiment I	Experiment II.	Experiment I	Experiment II.
		mg per cent	mg per cent	mg per cent	mg. per cent
21	0	11 0		3 0	
24	3	5 5	7 0	16 0	14 5
28	7	4 6	8 8	14 7	12 6
31	10	8 0	9 6	13 5	11 6
35	14	7.5	8 9	6 5	8 8

Results.

1. *Blood Serum Analyses.*—The blood findings of the rats at 21 days were typical of rickets—normal or slightly elevated calcium and low phosphorus of the serum. 3 days after the addition of phosphate a remarkable change had taken place. The calcium became low and the phosphorus very high. After 2 weeks on the same diet both tended toward the values normal for the rat. These results were corroborated in two independent repetitions of the experiment.

The results are given in Table I and Fig. 2. Beginning with normal values of 10 mg. of Ca and 9 mg. of P per 100 cc. of serum the phosphorus in the control group drops to 3 to 4 mg. at the 3rd

week and remains at this level. The calcium remains normal or slightly elevated. When phosphate is added to the diet, the blood serum changes markedly and rapidly. On the 3rd day the P rises to 14.5 to 16 mg. per 100 cc. and the Ca falls to 5.5 to 7 mg. Both calcium and phosphorus tend later toward normal and reach normal in 2 weeks.

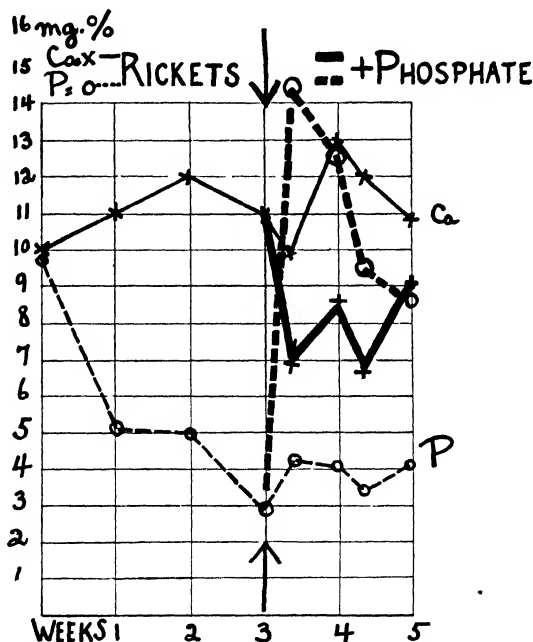


FIG 2 Effect of added P on serum of rickets rats. The arrow indicates the time at which phosphate was added to the rickets-producing diet to make the ratio of Ca P = 10.

The data unquestionably measure a profound change in the animal. Values so high in phosphorus have not been previously observed in rickets animals, nor in normal animals when large doses of phosphorus are given by mouth (Salvesen, Hastings, and McIntosh (4)). The high phosphorus and corresponding drop in the calcium values would seem to be the direct result of the addition of phosphate as in Binger's (5) experiments when phosphate was given intravenously. With such high phosphorus values

and low calcium one would expect tetany to supervene. In absence of complete blood analyses and electrical reactions of tetany and convulsions, positive diagnosis cannot be made. However, the fact that one animal died suddenly, in a manner not otherwise accounted for, and that three were found in carpopedal spasm, is strong evidence of tetany. The relations of calcium and phosphorus in the blood that determine whether bone can be laid down are just beginning to be understood (6-9). The high product of $\text{Ca} \times \text{P}$ indicates a condition suitable for rapid deposition of lime salts in bone.

2. *Histologic Examination of Bones.*—The histologic evidence bears out the blood findings. In the animals receiving additional phosphorus rapid calcification occurred. In the one animal killed and examined 24 hours after the addition of phosphorus to the diet, and in the other killed 48 hours afterwards, rapid deposition of lime salts had already begun. At 10 days the healing was already far advanced (three plus according to our notation).

3. *Analyses of Bones.*—The data represent calcium analyses on the air-dried bones. In the previous study the calcium on a rickets-producing diet was found to be 14 to 15 per cent in contrast to a normal value at 65 days of 21.1 per cent. When these animals were changed to the diet with added phosphate for 1 week the calcium was found at 65 days to equal 17.0 per cent. This indicates some further calcification, but less than that for normal bones at the same age.

4. *Metabolism of Calcium and Phosphorus.*—For the first 21 days the net amounts eaten are quite similar to those previously reported (1) on this high calcium-low phosphorus diet and are 4.75 compared to 4.4 gm. per rat per day. When phosphate was added, the rats did not eat so well and during the 1st week averaged 1.9 gm. daily intake. During the 2nd week food consumption was normal and averaged 4.5 gm. daily per rat.

Coincident with the change in food there was a marked loss in weight. At the same time the character of the feces changed from the usual dark brown to white and remained white as long as phosphate was added to the diet.

The dry weights of the stools are given in Table II and show consistent weekly amounts. The output of dry feces per rat per week was 3.8 gm. The weight of feces decreased by two-thirds during

the 1st week when phosphate was added and is in proportion to the food consumption. The change to a white color was associated with the alteration in composition of the feces (shown in Table III).

Discussion of Diet.

Our purpose was to select that diet which when given to ricketic animals would produce optimal bone deposition. We chose the diet especially with regard to the amounts and ratios of calcium and phosphorus. It neither contains vitamin D nor has it been irradiated, for these factors exert a "regulatory effect" (10) in the presence of disproportion of calcium and phosphorus. The

TABLE II
Weight of Dried Stools of Six Rats on Rickets-Producing Diet and Added Phosphate.

Expressed in terms of one rat per week.

Days of experiment	Days on added phosphate	Weight.
		<i>gm</i>
1-7		3.7
7-14		3.8
14-21		3.9
21-28	7	1.25
28-35	14	3.7

amount and ratio of calcium and phosphorus in the experimental diet after the addition of NaH_2PO_4 correspond very closely to that of McCollum's Diet 3173 (3), which contains 0.962 per cent of calcium and 1.0277 per cent of phosphorus, in the ratio 1:1.0683. The ratio of 1.0 was chosen because at the time the experiments were performed it was the best empirical value available.

Paths of Excretion.

For the fore period the per cent of calcium and phosphorus found in the urine and stools is similar to that found in the previous study (1). The calcium in the urine averages 19.5 per cent of the calcium excretion or 15 per cent of the intake. The calcium in the feces averages 80.5 per cent of the calcium excretion or 63 per cent of the intake. The phosphorus in the urine equals 6.5 per cent of

the excretion or 5 per cent of the intake. The phosphorus in the feces amounts to 93.5 per cent of the excretion or 68 per cent of the intake.

When phosphate was added to the diet this condition was considerably altered. The calcium in the urine diminished in actual amount and relatively to the intake. The calcium in the feces increased very slightly in per cent of the excretion. The phosphorus in the urine became much increased, both relatively and absolutely. The phosphorus in the feces increased absolutely and decreased relatively.

The actual values are calcium in urine equals 8 and 12.5 per cent of the excretion and 5 and 10 per cent of intake. The calcium in stool amounts to 92 and 87 per cent of excretion and 56 and 72 per cent of intake. The phosphorus in the urine equals 63 and 47 per cent of the excretion and 49 and 37 per cent of the intake. The phosphorus in the feces represents 37 and 53 per cent of the excretion and 29 and 42 per cent of the intake.

The addition of phosphate to the diet shifted some of the calcium from the urine to the feces. Phosphorus excretion was shifted in the opposite way. In the fore period 6.5 per cent of the phosphorus excretion was in the urine. In the two later periods 63 and 47 per cent were thus excreted. Thus in the 1st week of added phosphate, though the intake was nearly doubled, there was actually a smaller amount of phosphorus in the feces.

The results are more striking when the ratios of Ca:P in the urine and feces are considered. In the urine the ratio of Ca:P = 13.7 for the fore period changes to 0.095 and 0.26 during the administration of phosphate. In the feces the ratio of 3.9 during the fore period becomes 1.8 and 1.6 in the last 2 weeks. With diets such as we used Telfer (11) found that the ratios are similar whether or not a healing agent is present.

Balance of Calcium.

Positive balances of calcium occur both before and after the addition of phosphate. The data in Table III show that the average balance is greater before than after the alteration in diet: an average of 80 mg. per rat per week for the first three periods and only 55 to 58 mg. in the last two weekly periods. Both are considerably below normal; the former represents 50 per cent of the

normal and the latter 35 per cent. When the calcium retention is considered in per cent of the intake the conditions are altered; in the first 3 weeks the average retention is 22 per cent of the intake compared with 39 and 17.5 per cent retention during the added phosphate periods.

TABLE III.

Metabolism of Six Rats for Calcium and Phosphorus on High Calcium-Low Phosphorus Diet with Addition of Phosphate.

Figures in terms of one rat per week

Period	Calcium						Phosphorus					
	Intake	Urine	Feces	Total output	Balance		Intake	Urine	Feces	Total output	Balance	
<i>wks</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent of intake</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent of intake</i>
1		51	201	252	(+109)*			7	55	56	(+29)*	
2		26	246	272	(+89)*			8	58	66	(+19)*	
3		87	231	318	(+43)*			2	60	62	(+23)*	
Total for 3 periods.	1083	164	678	842	+241		255	11	173	184	+71	
Average per wk.	361	55	226	281	+80	22	85	4	58	62	+23	28
+PO ₄												
4	142	7	80	87	+55	39	150	74	44	118	+32	21
+PO ₄												
5	330	34	238	272	+58	17.5	350	130	149	279	+71	20

* The values in parentheses represent the calculated balance when the food is allotted in equal amounts for each week.

Further the assumption was made that during the first 3 weeks the food could arbitrarily be allotted in equal amounts. When the metabolism is so calculated, the addition of phosphate results not only in a retention of a greater percentage of the calcium intake, but an actual increase in the amount of calcium retained over the previous weekly balances. Subsequent experi-

ments with weekly food intakes² have shown that the food intakes decrease progressively and hence justify the assumption that the retention of calcium decreases week by week.

The small positive calcium balances during healing may seem the weakest link in the evidence presented. This condition is brought about primarily by the poor nutrition of the animals when phosphate was added. Larger food intakes on a better diet would in all probability have resulted in larger balances. If our criteria of the healing of rickets are accepted, it is necessary to conclude that for healing, increased calcium balances are not essential. Healing of the ricketic process in the ricketic metaphysis is not associated primarily with the amount of the calcium and phosphorus balances, but with the changes which occur in the amounts of these substances in the blood serum, bones, and tissues, in the intermediary metabolism.

Balance of Phosphorus.

The balances of phosphorus also are given in Table III. In the period during which rickets was developing 28 per cent of the phosphorus was retained. When phosphorus was added the per cent of intake retained was diminished to 22 and 21, but as the intake in the last two periods was greater, the balances are larger. In the period of developing rickets the phosphorus retentions per rat per week averaged 23 mg; in the added phosphate periods 32 and 71 mg. If the average of the first periods represents 23 per cent of the normal, the added phosphate periods represent 32 and 71 per cent of the normal phosphorus retention.

In contrast to the calcium retention, the phosphorus retention was largely increased upon the addition of phosphate, so that the actual retention was 2 or 3 times that in the fore period. Only one-quarter of the phosphate eaten was retained; the remainder was excreted although the body was in a state of phosphorus starvation. In the presence of a large amount of calcium in the diet the phosphorus balance was not only below normal but was also relatively less than the calcium balance. After the addition of phosphate to the diet the phosphorus retention was increased absolutely and relatively more than the calcium retention.

² Unpublished data.

Ratio of Calcium and Phosphorus Retentions.

In the previous study the values given for the ratio of the retentions of Ca:P were 1.58 for the normal and 3.9 for the ricketic rats. For the first three periods in this study the ratio of Ca:P retained is $80:23 = 3.5$, which is in agreement with the previous value. This high ratio was interpreted to mean a relative deficiency in the phosphorus retention.

During the 2 weeks when phosphate was added to the diet this condition is altered. The ratios of 55:32 and 58:71 equal values of 1.7 and 0.8. This indicates that for the 1st week normal proportions of calcium and phosphorus were retained and for the 2nd week a large relative excess of phosphorus.

Discussion of Balances.

If our analysis of calcium-phosphorus metabolism is correct, the ratio of retentions is of greater significance than either balance alone. In the absence of a sufficient regulatory factor such as light or cod liver oil, the ratio of the retention determines whether rickets is progressing or is healing. The ratio Ca:P retained is primarily determined by the ratio of the same elements in the diet. This is corroborated by Orr, Holt, Wilkins, and Boone (14).

DISCUSSION.

The amount and ratio of Ca:P in the food and their effect upon the healing of rickets have been considered. Alteration of their ratio from 4.25 to 0.905 by the addition of phosphate was the single variable introduced. Other constituents of the diet were not modified nor was light or vitamin supplied. The effect of this single change may, however, be interpreted as due to (1) the increase in phosphorus, (2) the alteration of the acid-base value, or (3) the resulting small food intakes.

The total amount of phosphorus ingested was altered and increased, especially in the 2nd week. The rickets-producing diet, however, contained phosphorus in excess of the amount Pappenheimer, McCann, and Zucker (15) stated to be sufficient for healing; i.e., above 135 mg. per 100 gm. of diet. Hence there was already a twofold excess above the phosphorus requirement of the rat and more could only be in the nature of a further luxus consumption.

The change in the acidity of the diets from alkaline towards acid has been maintained by Zucker (16) to result in healing. McClendon (17) has also reported that alkali added to a diet increased its power to produce rickets. Shelling³ has, however, reported the opposite. McCollum *et al.* (3) have reported the same degree of rickets on his diet whether it contained CaCO_3 or CaCl_2 in equivalent amounts. The wide variability of the acid-base properties of diets which produce rickets both clinically and experimentally is well known. It is probably of different importance for each diet considered. The addition of acid phosphate shifted the acidity of our diet from 530 cc. of 0.1 N alkali per 100 gm. of diet to 320 cc. of 0.1 N alkali. The diet was thus still markedly alkaline.

When the animals received the phosphate they limited their food intakes to less than one-half the usual amount. Starvation has been shown by McCollum, Simmonds, Shipley, and Park (18) to produce a zone of calcification—the line test. How far the results are due to insufficient food intake and how far to the diet consumed, is at present an open question. However, the addition of NaH_2PO_4 to the modified Steenbock diet results in extremely high phosphorus and low calcium values in the blood serum. The histologic pictures of rapid calcium deposition in bone, the changes in the composition of the bones, the alteration in the calcium and phosphorus metabolism, indicate a healing more rapid than that produced by light or cod liver oil.

CONCLUSION.

The following conclusions seem warranted:

1. The addition of phosphate to a rickets-producing diet of high calcium-low phosphorus content, with vitamin and light factors unchanged, so as to bring the ratio of $\text{Ca:P} = 1:1$, causes a rapid healing of rickets in rats.

2. This is evidenced by (1) blood serum analyses, (2) histology of bones, (3) composition of bones, and (4) metabolism of calcium and phosphorus.

- a. The changes are from rickets toward tetany with extremely high phosphorus and low calcium in serum..

³ An unpublished thesis presented June, 1925, to the School of Medicine, Yale University, for the degree of Doctor of Medicine.

b. The metaphyses show more rapid calcium phosphate deposition than by any other method of curing rickets in rats.

c. The bones show small increases in the per cent of calcium.

d. The increased per cent retention of calcium, and the increased positive phosphorus balances, which result in a more nearly normal ratio of the calcium and phosphorus retained, are evidence that the metabolism is that of healing rickets.

3. Whether active rickets is present depends not upon the balance but upon the intermediary metabolism.

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THE INFLUENCE OF SOLVENT AND OF CONCENTRATION ON THE OPTICAL ROTATION OF THE PENTACETATES OF GLUCOSE AND MANNOSE.

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It has been known for a long time that the magnitude of the optical rotation of a given substance is a variable depending upon external conditions. Among these, concentration, solvent, and temperature are of major importance. Comparing the rotations of two epimeric substances containing in their molecules only 1 asymmetric carbon atom, it is found that the numerical values of the rotations of each epimer are identical for a given solvent and for a given concentration and that the differences are only in the direction of the rotation, one epimer rotating to the right and the other to the left. The question arises as to the behavior of substances with more than 1 asymmetric carbon atom, such as sugars. It was shown by Hudson¹ that the numerical values of the rotations of certain sugar derivatives may be regarded on the basis of van't Hoff's superposition theory as the algebraic sum of the rotations of the individual carbon atoms. Hudson has based on this conception a method of differentiation between the α and β forms of sugars and Levene² later showed that the same conception may serve as a basis for a method of differentiating between the configurations of individual carbon atoms of a pair of epimeric sugar acids. Observations on the α and β forms of sugars have brought to light the exceptional behaviour of some sugars, and in recent years Hudson³ and also Levene⁴ have made an effort

¹ Hudson, C. S., *J. Am. Chem. Soc.*, 1909, xxxi, 66.

² Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 146.

³ Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1424.

⁴ Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxxvii, 759, 771.

to connect the abnormal optical behavior with the peculiarities of the lactal structure of the exceptional sugars. However, before proceeding further in this direction it seemed desirable to answer the following question: Are the rotations of each asymmetric carbon atom of a molecule influenced by a given solvent in the identical manner or does the influence vary from carbon to carbon atom depending upon the configuration of the entire molecule or upon the differences in ring structure? Some suggestion as to the possibility of individual influence of the solvent on each carbon atom may be found in the observation of Levene and Meyer⁵ on the influence on rotation of the methylation of the individual carbon atoms of gluconic acid.

The present investigation deals with the influence of solvents and of concentrations on the rotations of the pentacetates of glucose and of mannose. These two sugars were selected for the reason that one (glucose) behaves normally according to the rule of Hudson and the second abnormally, whereas structurally they are a pair of epimers

The rotation of glucose pentacetate has already been measured in several solvents by Hudson and Dale⁶ Their observations were limited to low concentrations In the present investigation the concentrations were varied from about 2 to 80 per cent where solubility permitted.

The results are tabulated in Table I and are graphically represented by curves in Figs 1 and 2. In Columns 1, 3, and 5 are given the concentrations in gm per cc., the rotations being measured in tubes of 1, 2, and 4 dm., depending upon the sugar and the concentration It was so planned that in no case was the total rotation less than 7°. The numerical values of observed rotations divided by the length of the tube are given in Columns 2, 4, and 6 of Table I

These results are shown graphically in Figs 1 and 2 where the concentrations as given in Table I are plotted as abscissæ and the rotations in degrees per 1 dm tube as ordinates

From these curves, plotted on a large scale (commensurate with the experimental precision), the rotations at round concentrations

⁵ Levene, P A, and Meyer, G M, *J Biol Chem*, 1925, lxx, 535

⁶ Hudson, C S, and Dale, J K, *J Am Chem Soc*, 1915, xxxvii, 1264.

TABLE I.

Optical Rotations of the α - and β -Pentacetates of Mannose and Glucose in Different Solvents and at Different Concentrations.

$t = 25.0^\circ \pm 0.1^\circ$

$\lambda = 5461 \text{ \AA}$

$l = 1 \text{ dm.}$

Chloroform		Acetone.		Benzene	
Concentration in gm per cc $\times 10^3$	α	Concentration in gm per cc $\times 10^3$	α	Concentration in gm per cc $\times 10^3$	α
(1)	(2)	(3)	(4)	(5)	(6)
A α -Mannose pentacetate.					
	degrees		degrees		degrees
2.913	1.88	2.306	1.33	6.03	4.23
4.300	2.77	3.520	2.12	8.03	5.64
7.750	5.12	6.480	3.79	9.12	6.39
8.550	5.64	7.440	4.36	13.41	9.38
9.562	6.33	14.35	8.65	14.84	10.40
15.66	10.32	14.80	8.92	21.29	14.77
18.40	12.05	22.49	13.55	25.03	17.28
18.60	12.15	26.57	15.96	29.58	20.06
19.90	12.99	31.41	18.68		
24.10	15.30	33.29	19.64		
30.95	19.18	35.35	20.91		
45.80	27.60	49.98	28.76		
60.78	36.08	68.71	38.99		
80.33	47.16				
B α -Glucose pentacetate					
	degrees		degrees		degrees
5.96	6.95	5.28	6.59	5.67	6.32
7.58	8.86	8.04	10.08	8.81	9.01
9.42	11.00	8.89	11.14	9.56	10.66
11.50	13.42	13.07	16.41	13.46	15.04
14.85	17.32	16.41	20.68	15.15	16.89
20.12	23.48	19.69	24.74	17.66	19.84
25.28	29.68	24.22	30.49	19.73	22.31
29.90	35.55	27.93	35.08	26.15	29.88
31.00	36.35	31.28	39.34	28.26	32.20
36.78	43.60	34.65	43.56		
43.19	51.36	38.67	48.33		
C β -Mannose pentacetate.					
	degrees		degrees		degrees
2.111	-0.59	5.054	-1.63	4.948	-1.77
9.944	-2.82	7.996	-2.61	7.142	-2.54
10.73	-3.05	11.27	-3.68	9.83	-3.49
22.86	-6.53	13.49	-4.42	15.56	-5.32
28.70	-8.19	20.96	-6.85	16.27	-5.54
37.37	-10.71	27.79	-9.04	20.01	-6.76
42.87	-12.35	34.70	-11.22	27.94	-9.33
58.77	-16.97	43.62	-14.07		
		44.89	-14.46		

TABLE I—*Concluded*

Chloroform		Acetone		Benzene	
Concentration in gm per cc $\times 10^2$	α	Concentration in gm per cc $\times 10^2$	α	Concentration in gm per cc $\times 10^2$	α
(1)	(2)	(3)	(4)	(5)	(6)
D β -Glucose pentacetate					
	<i>degrees</i>		<i>degrees</i>		<i>degrees</i>
5 28	0 29	6 346	0 48 ₅	8 36	4 00
7 832	0 42 ₅	6 661	0 50	9 86	4 70
10 99	0 59	9 67	0 73	12 05	5 07
14 87	0 80	14 93	1 15		
18 70	0 99 ₅	18 39	1 46		
31 26	1 75	19 24	1 53		
35 10	2 02	24 79	2 01		
41 69	2 48	26 82	2 14		
46 19	2 80	31 59	2 61		
Glacial acetic		Methyl alcohol		Pyridine	
Concentration in gm per cc $\times 10^2$	α in degrees per 1 dm tube	Concentration in gm per cc $\times 10^2$	α in degrees per 1 dm tube	Concentration in gm per cc $\times 10^2$	α in degrees per 1 dm tube
E α -Mannose pentacetate.					
7 492	4 75	7 102	4 54	6 700	3 76
8 432	5 30	8 352	5 36	8 265	4 64
9 281	5 84	9 701	6 21	9 325	5 24
α -Glucose pentacetate					
7 852	9 78	6 645	8 01	6 672	7 16
8 315	10 32	8 472	10 20	8 363	8 98
9 244	11 48	9 045	10 83	9 662	10 39
β -Mannose pentacetate.					
Concentration in gm per cc $\times 10^2$	α in degrees per 2 dm tube	Concentration in gm per cc $\times 10^2$	α in degrees per 2 dm tube	Concentration in gm per cc $\times 10^2$	α in degrees per 2 dm tube
8 528	-5 32	6 796	-4 28	5 961	-4 72
9 610	-6 06	7 963	-4 86	7 009	-5 60
10 555	-6 68	9 601	-5 84	8 404	-6 65
β -Glucose pentacetate.					
Concentration in gm per cc $\times 10^2$	α in degrees per 4 dm tube			Concentration in gm per cc $\times 10^2$	α in degrees per 4 dm tube.
7 939	1.74			7 081	-0 97
8 007	1 74			8 097	-1 21
9 402	4 08			10 65	-1 45

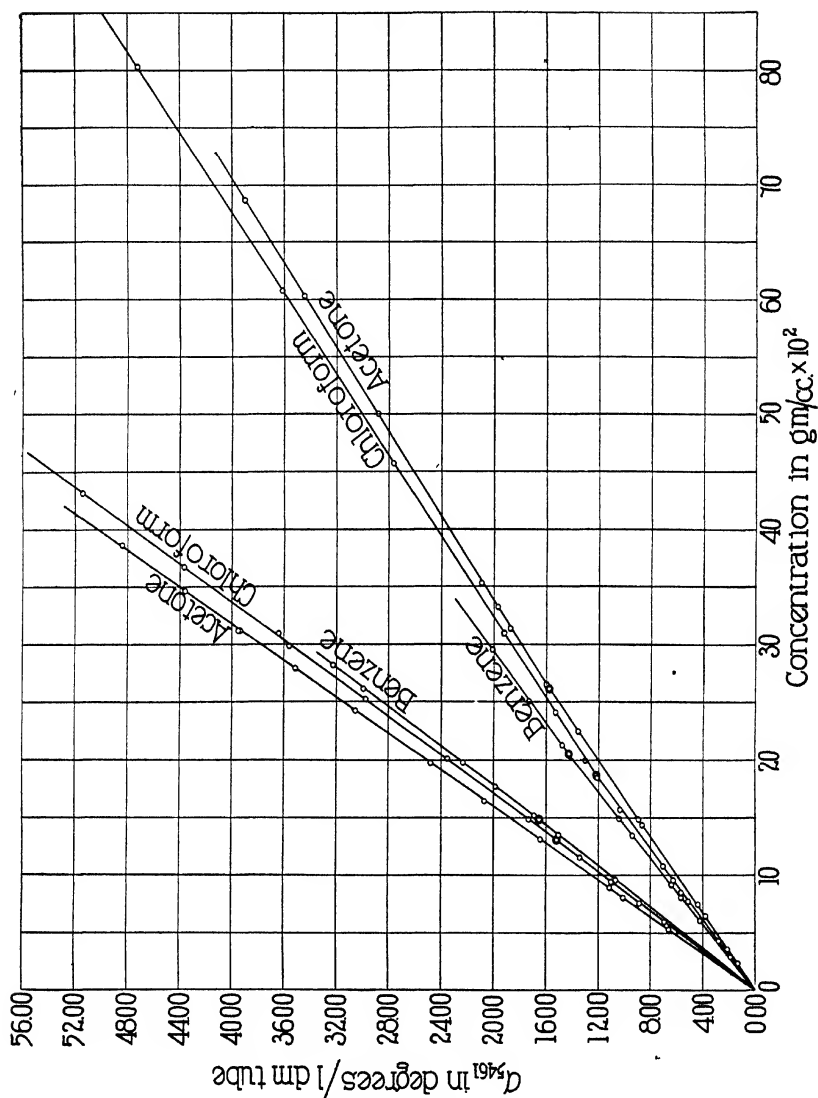


FIG. 1. Optical rotations of the pentacetates of α -glucose (upper group), and α -mannose (lower group) in different solvents. In Figs. 1 and 2 the double circles indicate points of inflection; not all of these are experimentally determined points.

were interpolated. The values thus obtained are given in Columns 2, 5, and 8 of Table II. The specific and the molecular rotations calculated from these values are recorded in the columns headed $[\alpha]$ and $[M]$ respectively.

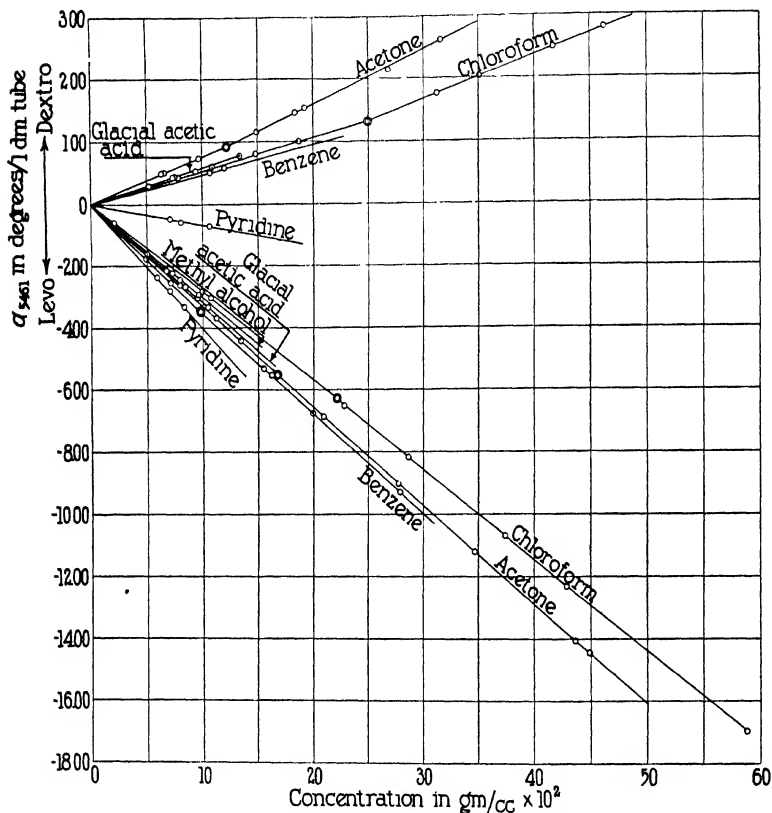


FIG 2 Optical rotations of the pentacetates of β -glucose (upper five curves) and β -mannose (lower six curves) in different solvents.

The differences between the α and β forms of the same sugar in the same solvent and concentration are given in Table III.

The analysis of the curves representing the rotations of the solutions as a function of concentration reveals certain very significant peculiarities. First, practically all the curves show at a certain concentration a break, after which the curve assumes a new

slope. Second, the curves expressing the rotations in individual solvents do not run parallel to each other, but diverge with increase in concentration. Third, the specific rotations remain practically constant in dilute solutions and change markedly with increase in concentration. This point is shown in Fig. 3 where the molecular rotations are plotted as ordinates and the concentrations as ab-

TABLE II.

Rotatory Power at Round Concentrations. $t = 25.0^\circ \pm 0.1^\circ$ $\lambda = 5461 \text{ \AA}$ $l = 1 \text{ dm.}$

Concentration in gm per cc $\times 10^2$ (1)	Chloroform			Acetone			Benzene		
	α (2)	[M] (3)	$[\alpha]$ (4)	α (5)	[M] (6)	$[\alpha]$ (7)	α (8)	[M] (9)	$[\alpha]$ (10)
A α -Mannose pentacetate									
2 0	1 28	250 ₀	64 0	1 12	218 ₄	56 0	1 40	273 ₀	70 0
4 0	2 50	243 ₈	65 5	2 34	228 ₁	58 5	2 81	274 ₀	70 3
6 0	3 90	253 ₅	65 0	3 57	232 ₀	59 5	4 21	273 ₆	70 2
8 0	5 22	254 ₅	65 3	4 77	232 ₅	59 6	5 62	274 ₀	70 2
10 0	6 52	254 ₄	65 2	6 00	234 ₀	60 0	7 07	275 ₇	70 7
12 0	7 86	254 ₉	65 5	7 21	233 ₈	60 0	8 41	272 ₇	70 7
14 0	9 17	255 ₄	65 5	8 42	234 ₆	60 1	9 82	273 ₆	70 1
16 0	10 45	255 ₄	65 3	9 62	234 ₅	60 1	11 20	273 ₀	70 0
18 0	11 69*	253 ₈	64 9	10 62	234 ₅	60 1	12 52	271 ₈	69 6
18 6	12 16	255 ₀	65 4	11 20	234 ₈	60 2	12 96	271 ₇	69 1
20 0	12 95	252 ₅	64 7	12 03	234 ₆	60 1	13 95	272 ₀	69 8
20 4							14 22*	271 ₈	69 7
22 0	14 08	249 ₆	64 0	13 23	234 ₅	60 1	15 23	270 ₀	69 2
24 0	15 21	247 ₂	63 4	14 44	234 ₆	60 2	16 52	268 ₅	68 8
25 0	15 72	250 ₉	62 8	15 04	234 ₆	60 2	17 14	267 ₄	68 5
26 1				15 75*	235 ₃	60 3			
30 0	18 62	242 ₁	62 0	17 81	231 ₉	59 5	20 36	264 ₄	67 9
35 0	21 46	239 ₁	61 3	20 58	229 ₈	58 8			
40 0	24 26	236 ₅	60 6	23 31	227 ₈	58 3			
45 0	27 14	235 ₂	60 3	26 05	225 ₈	57 9			
50 0	29 96	233 ₇	59 9	28 78	224 ₅	57 6			
60 0	35 65	231 ₇	59 4	34 21	222 ₄	57 0			
70 0	41 31	230 ₂	59 0						
80 0	46 90	228 ₆	58 6						

* The concentration at which there is an inflection in the curve.

TABLE II—Continued

Concentration in gm. per cc $\times 10^2$ (1)	Chloroform			Acetone			Benzene.		
	α (2)	[M] (3)	$[\alpha]$ (4)	α (5)	[M] (6)	$[\alpha]$ (7)	α (8)	[M] (9)	$[\alpha]$ (10)
B α -Glucose pentacetate									
2 0	2 30	448 ₅	115 0	2 50	487 ₅	125 0	2 23	434 ₈	111 5
4 0	4 67	455 ₈	116 7	5 00	487 ₅	125 0	4 46	434 ₈	111 5
6 0	7 03	456 ₉	117 2	7 56	487 ₅	125 0	6 69	434 ₈	111 5
8 0	9 37	456 ₈	117 1	10 03	488 ₉	125 3	8 94	435 ₈	111.7
10 0	11 68	455 ₁	116 8	12 54	489 ₁	125 4	11 16	435 ₂	111 6
12 0	14 00	455 ₀	116 6	15 06	489 ₄	125 5	13 38	434 ₈	111 5
13 05	15 20*	454 ₂	116 5						
14 0	16 31	454 ₃	117 0	17 59	490 ₀	125 6	15 61	434 ₈	111 5
16 0	18 68	455 ₈	116 7	20 14	490 ₉	125 9	17 88	435 ₈	111 7
18 0	21 04	455 ₉	116 8	22 64	490 ₅	125 8	20 25	438 ₇	112 5
20 0	23 40	456 ₃	117 0	25 14	490 ₂	125 7	22 60	440 ₇	113 0
22 0	25 79	457 ₂	117 2	27 64	490 ₀	125 6	24 93	441 ₉	113 3
24 0	28 20	458 ₁	117 5	30 15	489 ₉	125 6	27 27	443 ₁	113 6
26 0	30 60	459 ₀	117 7	32 68	490 ₂	125 7	29 60	444 ₀	113 8
28 0	33 00	459 ₆	117 9	35 18	490 ₀	125 6	31 90	444 ₈	113 9
30 0	35 42	460 ₄	118 1	37 66	489 ₆	125 5			
35 0	41 40	461 ₃	118 3	43 92	489 ₄	125 5			
39 0	46 29	462 ₉	118 7	48 96	489 ₆	125 5			
44 0	52 30	463 ₆	118 9						
C β -Mannose pentacetate									
3 0	0 85	110 ₅	28 3	0 96	124 ₈	32 0	1 08	140 ₄	36 0
5 0	1 41	110 ₀	28 2	1 62	126 ₄	32 4	1 79	139 ₆	35 8
7 0	1 97	109 ₈	28 1	2 28	127 ₃	32 6	2 48	138 ₅	35 8
9 0	2 54	110 ₁	28 2	2 95	127 ₈	32 8	3 20	138 ₇	35 8
9 9							3 48*	137 ₁	35 2
12 0	3 40	110 ₅	28 3	3 93	127 ₇	32 7	4 18	135 ₉	34 8
15 0	4 26	110 ₈	28 4	4 94	128 ₄	32 9	5 15	133 ₉	34 8
16 8				5 52*	128 ₁	32 6			
18 0	5 09	110 ₈	28 3	5 90	127 ₈	32 7	6 11	132 ₄	33 8
21 0	5 94	110 ₃	28 3	6 87	127 ₆	32 6	7 08	131 ₅	33 7
22 2	6 29*	110 ₅	28 3						
24 0	6 82	110 ₈	28 5	7 84	127 ₄	32 7	8 06	131 ₀	33 6
27 0	7 69	110 ₈	28 5	8 82	127 ₄	32 7	9 03	130 ₄	33 4
30 0	8 57	111 ₄	28 6	9 75	126 ₇	32 5	10 01	130 ₁	33 4
35 0	10 02	111 ₆	28 6	11 32	126 ₁	32 6			
40 0	11 49	112 ₀	28 7	12 90	125 ₈	32 2			
45 0	12 94	112 ₁	28 7	14 50	125 ₇	32 2			
50 0	14 40	112 ₃	28 8						
55 0	15 86	112 ₅	28 8						

TABLE II—Continued.

Concentration in gm per cc $\times 10^3$	Chloroform			Acetone			Benzene.		
	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)

D β -Glucose pentacetate

2 0	0 12 ₀	23 4	6 0	0 15 ₀	29 2	7 5	0.09 ₅	18 5 ₀	4 7
4 0	0 22 ₅	21 9	5 6	0 30 ₂	29 4	7 5	0 19 ₀	18 5 ₀	4 7
5 0	0 28 ₀	21 8	5 6	0 37 ₅	29 2	7 5	0 24 ₀	18 7 ₀	4 8
7 0	0 38 ₅	21 4	5 5	0 52 ₅	29 2	7 5	0 33 ₅	18 6 ₅	4 9
9 0	0 48 ₀	20 8	5 3	0 68 ₀	29 4 ₇	7 5	0 43 ₀	18 6 ₅	4 8
10 0	0 54 ₀	21 0	5 4	0 75 ₀	29 2	7 5	0 48 ₀	18 7 ₂	4 8
12 0	0 64 ₅	21 0	5 4	0 90 ₀	29 2	7 5	0 57 ₅	18 6 ₉	4 8
12.2				0 92 ₀ *	29 4	7 5			
14 0	0 74 ₅	20 8	5 3	1 07 ₁	29 8	7 6	0 66 ₅ *	18 5 ₂	4 7
16 0	0 85 ₅	20 8	5 3	1 24 ₄	30 3	7 8			
18 0	0 96 ₀	20 8	5 3	1 42 ₀	30 7 ₇	7 9			
20 0	1 06 ₀	20 6 ₇	5 3	1 59 ₀	31 1 ₈	8 0			
22 0	1 16 ₀	20 5 ₆	5 3	1 77 ₅	31 4 ₇	8 1			
25 0	1 32 ₀ *	20 5 ₉	5 3	2 03 ₅	31 7 ₅	8 1			
27 0	1 45 ₅	21 0 ₂	5 4	2 21 ₀	31 9 ₂	8 2			
29 0	1 59 ₅	21 4 ₅	5 5	2 38 ₂	32 7 ₈	8 2			
32 0	1 80 ₅	22 0 ₀	5 6	2 64 ₀	32 9 ₂	8 3			
35 0	2 02 ₀	22 5 ₁	5 8						
40 0	2 36 ₂	23 0 ₃	5 9						
45 0	2 71 ₃	23 5 ₁	6 0						

Concentration in gm per cc $\times 10^3$	Glacial acetic acid			Methyl alcohol			Pyridine		
	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$

E α -Mannose pentacetate

2 0	1 24	241 8	62 0	1 29	251 5	64 5	1 12	218 4	56 0
4 0	2 51	244 5	62 7	2 56	249 6	64 0	2 24	218 4	56 0
6 0	3 76	240 6	61 7	3 84	249 6	64 0	3 37	218 8	56 1
8 0	5 04	245 7	63 0	5 12	249 6	64 0	4 48	218 4	56 0
10 0	6 28	244 9	62 8	6 40	249 6	64 0	5 61	218 8	56.1

 α -Glucose pentacetate.

2 0	2 48	483 6	124 0	2 40	468 1	120 0	2 13	417.3	107.0
4 0	4 96	483 6	124 0	4 81	468 1	120 0	4 28	417 3	107 0
6 0	7 45	483 6	124 0	7 22	468 1	120 0	6 43	417 3	107.0
8 0	9 93	483 6	124 0	9 64	468 1	120 0	8 58	417 3	107.0
10 0	12 42	483 6	124 0	12 08	471 9	121 0	10 65	417 3	107 0

TABLE II—*Concluded*

Concentration in gm per cc $\times 10^2$ (1)	Glacial acetic acid			Methyl alcohol			Pyridine		
	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$	α	[M]	$[\alpha]$
	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
β-Mannose pentacetate									
2 0	-0 62 ₃	121 3	31 1	-0 60 ₈	118 5 ₅	30 4	-0 79 ₅	154 8	39 7
4 0	-1 25 ₆	122 4	31 4	-1 20 ₁	117 0 ₀	30 0	-1 58 ₂	154 4	39 6
6 0	-1 89 ₅	123 2	31 6	-1 82 ₇	118 5 ₅	30 4	-2 37 ₅	154 4	39 6
8 0	-2 53 ₀	123 2	31 6	-2 43 ₅	118 5 ₅	30 4	-3 16 ₂	154 1	39 5
10 0	-3 16 ₃	123 2	31 6	-3 05 ₀	118 9 ₆	30 5	-3 96 ₀	154 4	39 6
β-Glucose pentacetate									
2 0	0 22 ₁	42 90	11 0				-0 13 ₅	26 13	6 7
4 0	0 44 ₀	42 90	11 0				-0 26 ₂	25 74	6 6
6 0	0 66 ₂	42 90	11 0				-0 40 ₅	26 13	6 7
8 0	0 88 ₀	42 90	11 0				-0 54 ₈	26 52	6 8
10 0	1 10 ₀	42 90	11 0				-0 69 ₂	26 91	6 9

scissæ. Fourth, the numerical values of the specific rotation as a function of the solvent can be arranged in the following order.

A For the α and β forms of glucose pentacetate the order is: acetone > glacial acetic acid > methyl alcohol > chloroform > benzene > pyridine.

B The order for the pentacetates of mannose is, for the α form: benzene > chloroform > methyl alcohol > glacial acetic acid > acetone > pyridine; for the β form, chloroform > methyl alcohol > glacial acetic acid > acetone > benzene > pyridine.

In this connection emphasis should be placed on the fact that the order of solvent influence on rotation in the mannose pentacetates is different from that in the two glucose pentacetates, and furthermore, that it is different in each of the two mannose pentacetates. In the case of the two α forms, the order is the same, but in the opposite sense as shown in Fig. 4. An exceptional position is occupied by pyridine. In this solvent all forms show the lowest rotations.

Each of the four observations has its own significance. From the first observation one may conclude that the relationship between solvent and solute changes with increase in concentration.

As yet there do not exist sufficient data to offer a definite explanation of that relationship. It may be of a chemical nature, the solvent forming a complex with the solute. The ratio of the complex molecules to the simple may change with the increase in con-

TABLE III

Differences between $[\alpha]_{\text{M}}^{25}$ of α - and β -Mannose Pentacetates and α - and β -Glucose Pentacetates at Round Concentrations.

Concentration in gm per cc $\times 10^2$	Mannose pentacetates $[\alpha]_{\alpha} - [\alpha]_{\beta}$			Glucose pentacetates $[\alpha]_{\alpha} - [\alpha]_{\beta}$		
	Chloroform	Acetone	Benzene	Chloroform	Acetone	Benzene
3 0	92 3	89 3	106 0	110 2	117 5	106 8
5 0	93 7	91 7	106 0	111.4	117 5	106 8
7 0	93 6	92 1	105 7	111 6	117 6	106 8
9 0	93 4	92 6	105 7	111 7	117 8	106 9
10 0	93 4	92.8	105 6	110 4	117 9	106 8
12 0	93 8	92 8	104 9	110 3	118 0	106 7
14 0	93 8	93 0	104 3	111 7	118 0	106 7
16 0	93 7	93 0	104 3	111 4	118 1	
18 0	93 2	92 8	104 5	111 5	117 9	
20 0	93 7	92 7	103 6	111 7	117 7	
22 0	93 0	92 7	102 9	112 0	117 6	
24 0	91 9	92 9	102 4	112 2	117 5	
25 0	91 3	92 9	102 3	112 3	117 5	
30 0	90 7	92 0	101 3	112 5	117 3	
35 0	89 9	91 4		112 5	117 2	
40 0	89 3	90 5		112 8		
45 0	89 0	90 1		112 8		
50 0	88 7					
55 0	88 4					
	Glacial acetic acid		Pyridine	Glacial acetic acid		Pyridine
2 5	62 4		96 0	113 6		112 8
5 0	62 8		95 6	112 8		113 2
7 5	62 6		95 4	112 6		112 6
10 0	62 6		95 4	113 0		114 0

centration. It may also be assumed that the solvent is attached to different atoms or groups of the solute depending upon the concentration. In fact, it has been shown that pentacetate of glucose forms a definite compound with benzene⁷ and also with

⁷ Moll van Charante, J., *Rec. trav. chim. Pays-Bas*, 1902, xxi, 42.

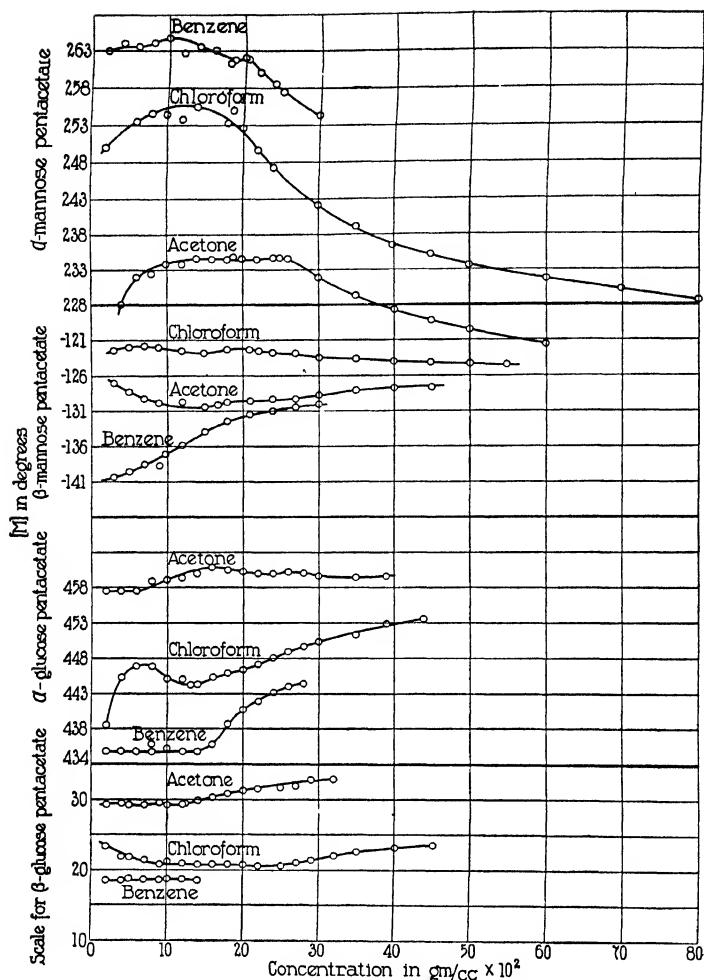


FIG 3 Molecular rotations of the pentacetates of α - and β -glucose and of α - and β -mannose in different solvents

α -Glucose	pentacetate in	benzene	[M] scale,	O K
"	"	chloroform	"	" add 10.
"	"	acetone	"	" " 30
β -Glucose	"	all solvents	"	" O K.
α -Mannose	"	benzene	"	"
"	"	acetone	"	" subtract 2.
"	"	chloroform	"	" " 12.
β -Mannose	"	acetone	"	" O K.
"	"	chloroform	"	"
"	"	benzene	"	" add 10.

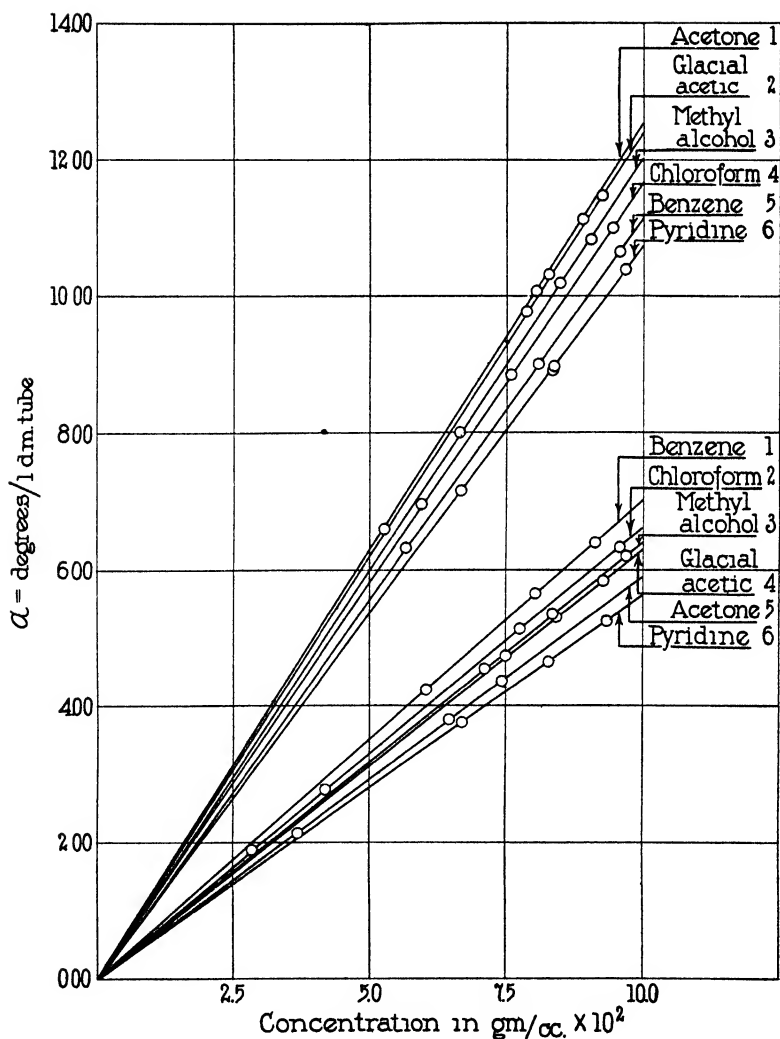


FIG. 4. The effect of solvents on the optical rotation of the pentacetates of α -glucose (upper group) and α -mannose (lower group).

pyridine.⁸ On the other hand, the influence of the solvent may be entirely external, affecting the degree of deformation of the solute.

From the second observation it may be concluded that each solvent forms a different complex with the solute or that it brings about a different form of distortion of the molecule of the solute.

The third observation needs no special discussion in the light of the first two. However, it emphasizes a practical point; namely, that in the comparative study of the numerical relationships of the rotations of different sugars, those series of concentrations should be selected in which the specific rotations remain constant.

On the fourth observation centers the principal interest of the present investigation. It demonstrates that there exists a divergence in the influence of the solvents on the pentacetates of glucose as compared with the influence on the pentacetates of mannose. The differences are marked not only in regard to the respective specific rotations, but also in regard to the differences in the rotations of the α and β forms.

Hudson⁶ and his coworkers have shown that with respect to the latter value the glucose pentacetates behave normally. In his latest publication Hudson³ assigns to them the $\langle 1,4 \rangle$ structure. In the same publication Hudson assigns to β -mannose pentacetate the $\langle 1,4 \rangle$ structure and referring to the α form he remarks: "For the present the acetate of $+55$ rotation will be left unclassified; the determination of its ring form and even the question whether it may not be a mixture of substances *remain outstanding problems*."⁹ Levene and Sobotka,⁴ on the other hand, on the basis of a comparative study of the rotations of the pentacetates of glucose, galactose, and mannose, were inclined to assign to the pentacetates of glucose the $\langle 1,4 \rangle$ ring and to those of mannose the $\langle 1,5 \rangle$ ring.

If a classification of pentacetates should be made on the basis of the order of influence of the solvent, the conclusion would necessarily be reached that the α - and β -glucose pentacetates belong to one type of structure, and that the α and β forms of mannose pentacetate belong to two different types, each distinct from that of the glucose pentacetates.

⁸ Behrend, R., *Ann. Chem.*, 1907, cccclui, 106.

⁹ The italics are ours.

On the other hand, if the differentiation of the pentacetates should be made on the basis of the differences of the molecular rotations of the α and β forms, then one will be confronted with the following confusing facts.

The ratios of $\frac{[M]_{\alpha} - [M]_{\beta} \text{ glucose pentacetate}}{[M]_{\alpha} - [M]_{\beta} \text{ mannose pentacetate}}$ differ in individual solvents in the following way: chloroform 0.84, acetone 0.78, pyridine 0.85, glacial acetic acid 0.55, benzene 1.0.

Thus, if the conclusion was based on an observation in a single solvent the classification would depend on chance. In benzene the differences of the rotations of glucose pentacetate and of mannose pentacetate have the same numerical value; in glacial acetic acid the divergence is the greatest. In the other solvents the values are approximately in the middle of the distance between the two extreme values.

Thus, it seems suggestive that both the α and β forms of mannose pentacetate belong to a different type from that of the glucose pentacetates and it is also possible that the two forms of mannose pentacetate have ring structures differing from each other as was suggested by Hudson.

More information on the influence of various solvents on the rotations of epimeric sugar derivatives is needed before a final conclusion can be reached as to the structural relationships of the pentacetates of glucose and mannose. As yet, the problem is not definitely settled.

EXPERIMENTAL.

A Schmidt and Haensch polarimeter supplied with a large direct vision spectroscope as a monochromator was used in this work. A quartz mercury lamp served as a source of light. The green line 5461 \AA was employed. The purity of this light, as well as the accuracy of the polarimeter, was tested by means of a quartz test plate recently calibrated at the Bureau of Standards. Jacketed tubes were employed throughout and a rapid stream of water from a regulated thermostat maintained a constant temperature of $25.0^{\circ} \pm 0.1^{\circ}$.

The solutions were made up by weighing the solids directly in 10, 15, or 25 cc. flasks. The solvent was then added up to the calibration mark. The flasks were carefully calibrated at 25.0° ,

and in making up the solutions the solvent was brought up to mark only after the solution and the flask had been allowed to come to the proper temperature. Extra pains were taken in making up these solutions inasmuch as the total volume of solvent was small and a slight error in the volume made appreciable discrepancies in the final result.

Throughout this research the same sample of substance was used in the case of α - and β -glucose pentacetates and β -mannose pentacetate. In the case of the α -mannose pentacetate, however, we were obliged to use several samples. From these, only those giving the highest rotations were used.

DETERMINATION OF UREA BY GASOMETRIC MEASUREMENT OF THE CARBON DIOXIDE FORMED BY THE ACTION OF UREASE.

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Urea is changed by the action of urease into ammonium carbonate: $\text{CO}(\text{NH}_2)_2 + 2 \text{H}_2\text{O} = (\text{NH}_4)_2\text{CO}_3$.¹ The ammonia has been more commonly determined as a measure of the urea. However, as shown by Partos (3) and by Mirkin (2), one can also obtain an exact estimate by determining the CO_2 of the ammonium carbonate. The manometric blood gas apparatus (7, 4) is particularly adapted to this determination, because of the wide range over which it yields accurate results. In practice the gasometric CO_2 urea determination has proved to have several advantages over the ammonia estimation. The gasometric method dispenses with the apparatus required for aeration or distillation of the ammonia, and with the necessity for exact standard solutions for titration, or for colorimetric comparison in Nesslerization. The result is a diminution in the sources and likelihood of error and, at least in the case of urine analyses, a gain in rapidity.

Preformed carbonic acid and bicarbonate exist in both blood and urine. This CO_2 is removed by acidifying and agitating the blood or urine before the urease is added. In analysis of urine the CO_2 is removed in a volumetric flask by acidifying with phosphoric acid and whirling the fluid about the walls of the flask. Sodium hydroxide is then added in amount to form the optimum phosphate buffer mixture for the action of urease. The urease is then added, with sufficient water to dilute the urine sample either 10-

¹ For experiments and literature concerning the mode of action of urease and the optimum conditions for its action see Van Slyke and Cullen (5) and Van Slyke and Zacharias (9).

or 20-fold. After the urease has acted 20 minutes samples of the solution may be drawn for CO_2 determinations. The latter, by the technique described below, can be easily performed in series at the rate of a determination every 4 minutes. This procedure has been in continual use for routine urine analyses in this laboratory for about a year.

A similar technique is applicable to the Folin-Wu filtrate of blood, and has proven satisfactory in routine hospital analyses. In this case it is convenient to let the urease and aerated filtrate react inside the gas apparatus, a procedure which prolongs the time for a determination only by the 1 minute required for the enzyme to act.

The removal of the blood proteins is not a necessary preliminary to the analysis. The determination can be performed directly on either whole blood or plasma. The only drawback is that the determination requires 15 minutes, compared with the 5 for the Folin-Wu filtrate. In the direct analysis of whole blood or plasma the preliminary removal of CO_2 is most conveniently performed in the gas apparatus itself. The blood sample is introduced, acidified with lactic acid, and freed of CO_2 by extraction *in vacuo*. Na_3PO_4 is added in such amount as to form an optimum phosphate buffer mixture; *viz.*, with $\text{Na}_2\text{HPO}_4 : \text{NaH}_2\text{PO}_4 = 1 : 1$ (9). The urease solution is added to the blood solution so prepared, and, with the enzyme we have employed, it has been necessary to wait only 1 minute for it to act, even with uremic blood. An excess of lactic acid is finally added, and the CO_2 is extracted and measured by observation of the gas pressure before and after absorption with sodium hydroxide.

In accuracy there is no difference between determination of the CO_2 and that of the NH_3 formed by the action of urease, if samples of size best suited for the measurement of each are taken. In urine analyses this is the case. In blood analyses the results obtainable with 1 cc. samples by the gasometric method are about equal in accuracy (1 per cent of the amount measured) to those obtainable with 5 cc. by the ammonia titration method, and considerably more accurate than those usually obtainable by titration with the 2 cc. samples commonly used. Gasometrically one can obtain with a little practice very satisfactory accuracy, by the micro technique described below, with 0.2 cc. of blood taken from an ear puncture.

*Determination of Urea in Urine.**Reagents.*

Urease Solution.—We have used a 10 per cent water solution of Squibb's urease, which is prepared from jack beans by Van Slyke and Cullen's acetone precipitation method (6). We have always found this of full activity and free of CO_2 . Any other CO_2 -free urease preparation may presumably be used, if the activity or the time required by it to decompose completely the maximum amount of urea encountered in human urine (3 per cent) is determined. The urease solution in 50 per cent glycerol prescribed for blood analysis may also be used for urine.

1 N Lactic Acid (Approximate).—Made with sufficient accuracy by diluting 1 volume of commercial lactic acid of 1.20 specific gravity to 10 volumes with water.

2 M H_3PO_4 .—Dilute 13.2 cc. of syrupy H_3PO_4 , of specific gravity 1.725, to 100 cc.

Saturated Carbonate-Free NaOH Solution.—Sodium hydroxide is dissolved in an equal weight of water and permitted to stand in a closed vessel until the carbonate has settled. The concentration of NaOH is approximately 18 N.

2 M Carbonate-Free NaOH.—Dilute 11.0 cc. of the saturated sodium hydroxide to 100 cc. At once draw the solution up into a soda-lime tube (Fig. 1) protected at the top by soda-lime from atmospheric CO_2 .

Brom-Thymol Blue, 0.4 Per Cent Solution.—100 mg. of the powdered dye are ground in a mortar with 3.0 cc. of 0.05 N NaOH, and then diluted to 25 cc. with water.

Caprylic-Ethyl Alcohol.—1 volume of caprylic alcohol is mixed with 4 volumes of 95 per cent ethyl alcohol. This mixture is used to prevent foaming.

Test of 2 M H_3PO_4 and 2 N NaOH.

A portion of 2 M H_3PO_4 may be titrated with the 2 N NaOH, using phenolphthalein as indicator, and titrating to a full red. Each cc. of phosphoric acid neutralizes 2 cc. of sodium hydroxide. A variation of 10 per cent may be permitted; i.e., 1 cc. of H_3PO_4 may neutralize from 1.80 to 2.20 of NaOH.

The solutions may also be tested as follows: To 20 cc. of water

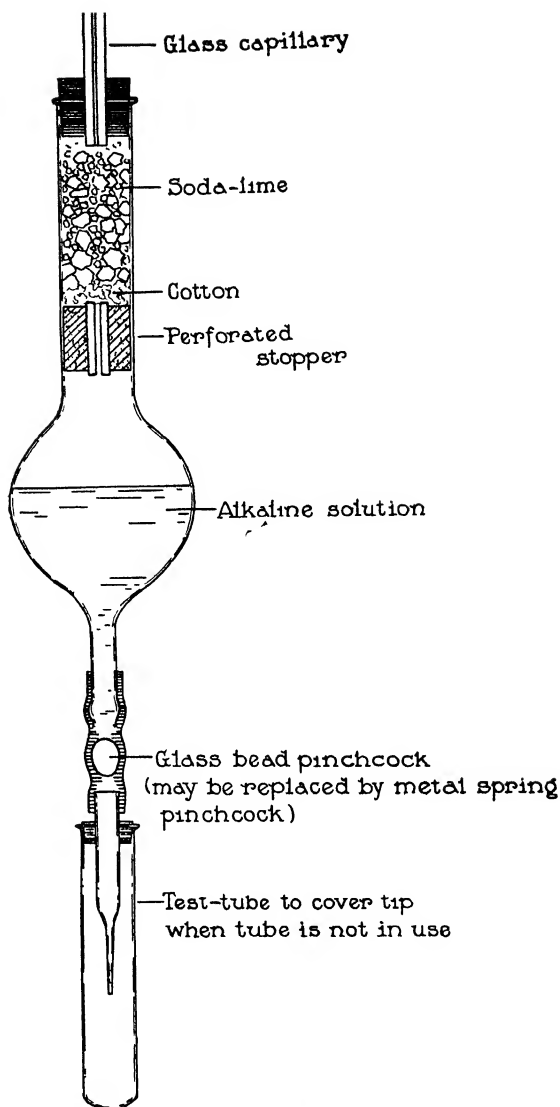


FIG. 1. Soda-lime tube of about 100 cc capacity for holding CO_2 -free alkali or alkaline phosphate. The tip is drawn out quite fine, so that it delivers 35 to 40 drops per cc, the exact number being determined by trial with the solution used and marked on the tube. The form of cock shown, made by a glass bead in a rubber tube, is convenient for delivery of drops by count. By pinching the tube about the bead delivery of the fluid is carried at the desired rate. An ordinary metal pinch-cock may also be used.

add 1 cc. of each solution. Mix and pour half into each of two test-tubes. To one add methyl orange indicator, to the other add sodium alizarin sulfonate. Both should become yellow, indicating that the solution is alkaline to methyl orange, acid to alizarin red, and is therefore at a pH between 4 and 5.

The 2 N NaOH should be titrated, or tested as above, when it is made up, and once a week thereafter. If the solution stands for some weeks or months in the soda-lime it is likely to increase in concentration by more than the permitted 10 per cent, as the result either of losing water by slow distillation up into the soda-lime, or of solution of more alkali from the glass. An intact coat of paraffin on the tube should prevent reaction with the glass. It is advisable even with a paraffin-coated tube, however, to test alkali that has stood in it for some time

Procedure for Urea Determination in Urine.

Removal of Preformed CO₂.—Place in a 20 cc. measuring flask 2 cc. of urine of specific gravity below 1.030, or 1 cc. of more concentrated urine. Add 0.25 cc. of 2 M H₃PO₄, which may be measured conveniently and with sufficient accuracy by drops delivered from the fine glass tip of a dropping pipette, and 3 drops of brom-thymol blue solution. Whirl the urine about the walls of the flask for 1 minute to permit CO₂ to escape. Insert a tube into the flask and draw air through it to sweep out the CO₂ gas. Then whirl the urine again for a minute to permit escape of the last traces of CO₂. (This second treatment is necessary only for urines with such high bicarbonate content that they are alkaline to litmus, but it is probably safer and as little trouble to carry it through as a routine procedure on all.)

Digestion of Urine with Urease.—Dilute to about 18 cc. Add 0.35 cc. of 2 N NaOH, measured by drops from the tube (Fig. 1) in which it is stored. (The first drops from the tube are discarded, as the solution at the capillary tip is likely to have taken up CO₂ from the air.) Then add 1 cc. of urease solution from a pipette which dips below the surface of the diluted urine. The heavy urease solution sinks to the bottom of the flask, and does not decompose the urea at the top until after the solutions are mixed. The solution is now diluted up to 19.9 cc., and the flask is closed with a 1-hole rubber stopper, the hole of which is filled by a vase-

lined glass rod (Fig. 2). The contents of the flask are mixed, and allowed to stand 20 or more minutes for the enzyme to act. The mixed solution should take on a distinctly green color, and may quickly become blue from formation of ammonium carbonate. If it does not change from yellow to at least green (pH about 6.5), add another drop of 2 N alkali through the hole in the stopper.

Addition of Excess Alkali to Digested Urine to Prevent CO_2 Loss.—At the end of the 20 minutes or longer period the glass rod is with-

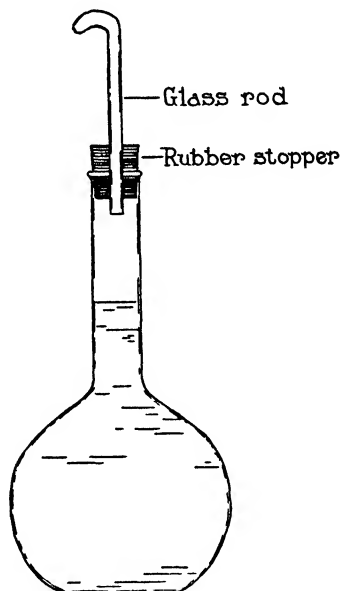


FIG 2. Volumetric flask for urine analysis. The lower end of the glass rod and the perforation through the stopper are vaselined before each analysis.

drawn from the stopper, and, after the first 3 drops of alkali have been discarded, the capillary tip of the tube (Fig. 1) delivering 18 N NaOH is inserted through the hole. 0.2 cc. of the concentrated alkali is added measured by (about 5) drops. It is advisable to vaseline the delivery tip, so that the alkali will fall cleanly from the end and not creep back up the sides. The 0.2 cc. of concentrated alkali increases the volume of the solution by only 0.1 cc. The stopper is again closed with the glass rod, and the flask is in-

verted 2 or 3 times to mix the alkali with the solution, and to absorb any CO_2 that may have escaped into the gas space beneath the stopper. The 0.2 cc. of concentrated NaOH makes the solution so alkaline that there is no danger of loss of CO_2 when the flask is subsequently opened for withdrawal of samples. To prevent absorption of atmospheric CO_2 , the flask is kept closed, except when samples are pipetted out for analysis.

Extraction and Measurement of CO_2 Formed from Urine Urea.—Place a drop of the caprylic-ethyl alcohol mixture in the capillary and 1 cc. of water in the cup of the 50 cc. blood gas apparatus of Van Slyke and Neill (4,7). Run 2 cc. of the digested urine solution under the water. Admit the urine solution into the chamber of the apparatus followed by the 1 cc. of water. Then add 0.5 cc. of 1 N lactic acid. Evacuate the chamber and shake $1\frac{1}{2}$ minutes. Reduce the gas volume to 2 cc. as described for CO_2 determinations in blood on p. 533 of Van Slyke and Neill's paper (7). Read pressure p_1 on manometer, and eject the solution from the apparatus.

Run a control with reagents alone, without any urine. The manometer reading for the control is p_0 .

The pressure due to CO_2 from urea is $P_{\text{urea}} = p_1 - p_0$.

One control serves for an entire series of urea determinations. If the temperature changes while the series is being analyzed, correct p_0 by adding or subtracting 1.3 mm. for each degree rise or fall of temperature centigrade. This serves to correct for the change in the vapor tension of the water in the apparatus, and in the pressure exerted by the air extracted from the solution, if the temperature does not vary more than 3° . If it does, repeat the analysis of the control solution.

Remarks on the Urine Analysis.

In running a series of analyses it is not necessary to wash the gas apparatus between determinations. Since the CO_2 is not reabsorbed with alkali in the apparatus, the ejection of the gas and the acidified solution after each analysis removes the CO_2 with sufficient completeness. One may consequently proceed with the analyses at the rate of one every 4 minutes, and have sufficient leisure to calculate each result during the extraction in the next analysis.

The 1 cc. of water and the 0.5 cc. of lactic acid solution added to the urine solution in the apparatus should be measured to within 0.05 cc. The dissolved air in the 3.5 cc. of solution is extracted and measured with the CO_2 , the correction for the air content being included in the blank. 0.1 cc. of water saturated with air at room temperature yields sufficient air to exert at 2 cc. volume about 1 mm. pressure in the apparatus, and it is desirable to keep the error within this limit. Hence the 3.5 cc. volume of water solution should be measured to within 0.05 cc. The 1 cc. of water first placed in the cup should be measured from a 1 cc. pipette, rather than by the mark on the cup itself.

Gasometric Urea Determination in Folin-Wu Blood Filtrate.

The determination may be made, as in the urine analysis described above, by measuring the total gas (air + CO_2) after the action of urease, and subtracting the dissolved air obtained in a blank analysis. This procedure (A) is the one of choice for speed and convenience when a number of blood urea determinations are required; each determination is simplified to one extraction and one reading of the manometer, and, as the CO_2 is not reabsorbed with alkali, the apparatus need not be washed between determinations. For the blank analysis, 0.9 per cent NaCl solution is used, because it dissolves the same amount of gas as the Folin-Wu filtrate; *viz.*, 94.5 per cent as much as does water.

When only one or two analyses are to be made, it is simpler to obviate the blank, and perform in the gas apparatus the extraction of preformed CO_2 , as well as the subsequent analysis. Accordingly both procedures will be described.

Reagents for Blood Filtrate Analysis.

0.9 per cent NaCl solution, acidified with 1 or 2 drops of 1 N hydrochloric or sulfuric acid per 100 cc.

0.5 M CO_2 -Free Na_2HPO_4 .—17.9 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$, or 7.1 gm. of anhydrous Na_2HPO_4 , made up to 100 cc. The solution should be made with minimum exposure to air, and at once drawn up into a soda-lime tube (Fig. 1).

Another way of preparation, from the H_3PO_4 and concentrated NaOH described for urine analysis, is also convenient. In a 100 cc. measuring flask place about 50 cc. of water and 3.33 cc. of syrupy H_3PO_4 of specific gravity 1.72 (this is approximately 15 M H_3PO_4).

Add 1 drop of phenolphthalein and run in 18 N NaOH till the solution turns pink. At once dilute to 100 cc., mix, and draw up into a soda-lime tube.

10 Per Cent Urease in Glycerol-Phosphate Solution.—Jack bean urease dissolved in water slowly produces carbon dioxide, sufficient in the course of an afternoon to increase by several millimeters the pressure reading in a blank analysis. When the enzyme is dissolved in 50 per cent glycerol CO_2 formation does not occur. Phosphate also is added to the solution, to neutralize the acidity of the Folin-Wu filtrate and provide a proper pH for the action of the urease.

Place 1 gm. of Squibb's urease in a 10 cc. measuring cylinder, and stir into it 2 cc. of water. When the mixture has become homogeneous add 3 cc. of the 0.5 M Na_2HPO_4 solution, followed by 5 cc. of glycerol. Mix, and at once draw up into a 10 cc. burette protected at the top by a soda-lime tube. The delivery tube of the burette below the cock should be 7 or 8 cm. long, and should be provided with a rubber tip to permit delivery through a mercury seal directly into the chamber of the gas apparatus, in the manner recently described (4).

5 N NaOH Solution.—27 cc. of the saturated NaOH solution, described for urine analysis, are diluted to 100 cc. and drawn into a soda-lime tube (Fig. 1).

1 N Lactic Acid (Approximate).—1 volume of commercial lactic acid of 1.20 specific gravity is diluted to 10 volumes with water.

0.4 per cent brom-thymol blue, described above for urine analyses.

Caprylic-ethyl alcohol mixture, 1 to 4, described for urine analyses.

Procedure A, for Determinations in a Series of Blood Filtrates.

Preliminary Aeration of Filtrate and Control Solution.—Prepare the filtrates as described by Folin and Wu (1).² Collect each fil-

² Instead of diluting the blood with water and then adding 1 cc. each of $\frac{2}{3}$ N sulfuric acid and 10 per cent sodium tungstate, a convenient simplification for routine analyses is to use a single solution containing the water, tungstate, and acid. It is prepared by adding 1 volume of the 10 per cent tungstate to 8 volumes of N/12 sulfuric acid. Enough of this is prepared to last about a fortnight. If it stands longer a precipitate is likely to form in it. In that case it is discarded. 1 volume of blood is mixed with 9 volumes of this solution.

trate in a flask of volume equal to at least 10-fold that of the filtrate, and add a drop of 0.4 per cent solution of brom-thymol blue for each 10 cc. The filtrate must show a clear yellow acid color. To remove preformed CO_2 derived from the blood bicarbonate, shake each flask, *unstoppered*, vigorously with a horizontal, whirling motion for 15 seconds. Insert a tube into the flask, and draw fresh air through it for a few seconds. Repeat this process twice more. Each extraction removes about 90 per cent of the CO_2 present, so that the last leaves only a negligible thousandth of that originally present. Several flasks may be held in the hand and shaken together, when a series of analyses is being performed.

Treat a similar volume of acidified 0.9 per cent NaCl solution in the same manner, with the precaution to ascertain that the solution is *within 0.2°C of the temperature of the filtrates*. A variation of 1° would change the dissolved air content by 0.003 volume per cent, and affect the obtained blood urea nitrogen content by 0.5 mg. per 100 cc.

Transfer of Sample to Gas Apparatus and Digestion with Urease.—With a 5 cc. pipette, provided with a rubber tip as described by Van Slyke and Neill, and calibrated to deliver between two marks, transfer 5 cc. of filtrate to the chamber of the blood gas apparatus. Before the transfer a small drop of caprylic-ethyl alcohol is drawn into the capillary below the cup, into which are then poured 3 or 4 cc. of water and 0.5 to 1 cc. of mercury. The tip of the delivering pipette is sealed by immersion in the mercury, so that the solution is delivered from the pipette into the chamber of the gas apparatus without danger of admixture with either air bubbles or water (4). It is convenient, though not obligatory, to use a pipette provided with a stop-cock.

0.5 cc. of the urease solution, measured from the burette, is then run into the cup under the mercury seal in the same manner. Before the tip of the burette is lowered into the mercury it is washed in the water in the cup to remove the drop of urease-phosphate at the tip, which may have absorbed some CO_2 from the air. The urease and filtrate are mixed by lowering and raising the mercury in the chamber a few centimeters, and are permitted to react for 1 or more minutes. 1 minute is sufficient with the Squibb's urease we have used, of which 100 mg. at pH 6.8 to 7.2 and a temperature of 20° will decompose 9.5 mg. of urea per minute. The

mixture should turn to a greenish color. If it remains yellow, because of unusual acidity of the filtrate, another 0.5 cc. of urease-phosphate solution may be added.

Determination of CO₂ Formed from Urea.—After the urease has acted 1 minute 0.20 cc. of 1 N lactic acid is run under the mercury seal into the chamber from a rubber-tipped burette in the same manner as the sample and urease. The chamber is evacuated and shaken 1½ minutes. The gas volume is reduced to 0.5 cc. for ordinary blood, but only to 2.0 cc. for blood with high urea content. The technique described on p. 533 of Van Slyke and Neill's paper for adjusting the gas volume in CO₂ determinations is to be followed. In case one attempts first to reduce the volume to 0.5 cc., but finds the gas pressure too high to read in the manometer, the mercury in the chamber must be lowered again to the 50 cc. mark, the solution shaken for another half minute, and the gas volume brought to 2.0 cc. With the gas volume at either 0.5 or 2.0 cc., the pressure p_1 is read on the manometer

5 cc. of 0.9 per cent NaCl solution are analyzed in the same manner. The reading is p_0

The pressure of CO₂ from urea is $P_{\text{urea}} = p_1 - p_0$ as in the urine analysis described above.

In case the temperature recorded by the thermometer in the gas apparatus changes during the interval between the analysis of the blank and that of the filtrate, 1.3 mm. is added to p_0 as in the urine analysis, for each 1° rise when the pressure is measured with the gas at 2 cc. volume. When the gas pressure is measured with the gas at 0.5 cc. volume the correction is 1.7 mm. per 1° temperature change. This correction is added to p_0 if the filtrate is analyzed with the gas apparatus at a higher temperature than the blank, and subtracted if the reverse occurs.

Procedure B, for Determinations in Single Samples of Blood Filtrate.

Removal of Preformed CO₂.—5 cc. of filtrate preceded by a drop of caprylic-ethyl alcohol are delivered into the gas apparatus under a mercury seal as described above. To remove CO₂ the chamber is evacuated and shaken 30 seconds. Mercury is admitted from below until the chamber is about one-quarter full. Then *without stopping the inflow of mercury, the upper cock is opened, admitting air into the chamber.* The admission of mercury into the chamber

is continued until all the gases have been completely driven out through the cock above. The air is admitted in order to dilute the CO_2 in the chamber and prevent its reabsorption by the blood solution. If the upper cock were kept closed until the extracted CO_2 had been compressed at the top of the chamber, some of the extracted CO_2 gas would go back into solution. The above procedure removes 90 per cent of the preformed CO_2 from the blood. It is repeated twice, making three extractions in all, which leave only a negligible 0.001 of the preformed CO_2 .

Digestion with Urease.—0.5 cc. of the urease-phosphate solution is measured into the cup of the apparatus, through a mercury seal, as in Procedure A. The urease is mixed with the filtrate in the chamber by once lowering the mercury to the bottom of the chamber. The mixture is allowed to stand 1 minute for completion of the action of the urease. It should turn from clear acid yellow to a green or blue color, indicating that the phosphate has neutralized the acidity of the filtrate. If the solution does not turn green, add a larger amount of urease-phosphate.

Determination of CO_2 Formed from Urea.—0.20 cc. of 1 N lactic acid is added through the mercury seal; the gases are extracted and their pressure, p_1 , read at either 0.5 or 2.0 cc. volume, as described above for Procedure A.

To absorb the CO_2 5 or 6 drops of 5 N NaOH are admitted into the chamber. The cock leading to the leveling bulb is then opened to permit the solution to rise for a moment into the upper stem of the chamber. The gas volume is brought again to the original 0.5 or 2.0 cc., and the manometer reading p_2 is taken. The pressure P_{urea} due to CO_2 formed from urea is

$$P_{\text{urea}} = p_1 - p_2 - c$$

where c is the value of $p_1 - p_2$ obtained in a blank analysis performed on 5 cc of acidified water in the place of blood filtrate. The c value, due to traces of CO_2 in the enzyme and phosphate solution, should be only a few millimeters with the gas volume at 0.5 cc.

Washing the Apparatus between Analyses.—Before each analysis the gas apparatus is washed twice with 5 to 10 cc. of distilled water, as described on p. 534 of Van Slyke and Neill's paper.

Direct Determination in Whole Blood or Plasma.

As in Procedure B described above for blood filtrate, the entire operation, including removal of preformed CO_2 and digestion with urease, is carried out in the gas apparatus. Two procedures will be described, for the analyses of 1.0 and 0.2 cc. samples respectively.

Reagents for Direct Analysis of Whole Blood or Plasma.

The reagents required are the 5 *N* NaOH and 1 *N* lactic acid, described above for analysis of the Folin-Wu filtrate, plus the following.

0.1 N Lactic Acid.—The approximately 1 *N* lactic acid is diluted 10-fold, and is standardized by titration against standard alkali. Water or 1 *N* lactic acid is then added in amount necessary to bring the concentration to 0.1 *N*. A margin of error of 3 per cent may be allowed.

0.02 N lactic acid (for micro blood analysis). The 0.1 *N* acid is diluted 5-fold.

0.26 M Na_3PO_4 Solution.—In a 100 cc. flask place 1.75 cc. of syrupy phosphoric acid of specific gravity $D_{20}^{20} = 1.72$ to 1.73.

This phosphoric acid is of approximately 15 *M* H_3PO_4 concentration. Fill the flask half full with water, add 2 drops of 1 per cent phenolphthalein solution, and titrate with the carbonate-free, saturated (approximately 18 *N*) NaOH solution from a 10 cc. burette until the solution in the flask turns red. At this point 2 molecules of NaOH have been added per molecule of H_3PO_4 , and Na_2HPO_4 has been formed. Note the volume of NaOH solution used and add exactly half as much more, to change the phosphate to Na_3PO_4 . The total volume of 18 *N* NaOH required should be about 4.3 cc. Immediately after the addition of the alkali has been completed, and before the alkaline phosphate solution has had opportunity to absorb CO_2 from the air, dilute the solution to the 100 cc. mark, stopper the flask, mix the solution, and draw it up into a soda-lime tube (Fig. 1), from which it may be measured by drops for analyses.

10 Per Cent Urease Solution in 50 Per Cent Glycerol.—1 gm. of Squibb's preparation of jack bean urease prepared by the acetone precipitation method of Van Slyke and Cullen (6) is dissolved in 5 cc. of water, and 5 cc. of glycerol are mixed with the solution.

The enzyme solution should be prepared the same day it is used. It need not be protected from air, as it is too acid to absorb CO_2 . It is not expedient to mix it with the Na_3PO_4 prior to use, because the alkalinity of the phosphate destroys the enzyme rather rapidly.

Determination in 1 Cc. of Whole Blood or Plasma.

Removal of Preformed CO_2 from Blood.—Place a drop of caprylic-ethyl alcohol in the cup of the blood gas apparatus, and draw it down into the capillary beneath the cup. Then place 1 cc. of 0.1 N lactic acid in the cup, and add water up to the 4.5 cc. mark. From a stop-cock pipette run 1 cc. of blood under the water solution into the chamber, and then draw the acidified water also into the chamber. If a bubble of air also enters the chamber no harm is done; it will be ejected later with the preformed CO_2 . The preformed CO_2 is now removed by three successive extractions of 30 seconds each, in the manner described above for Procedure B with the Folin-Wu filtrate.

Digestion of Blood with Urease.—After removal of preformed CO_2 , place in the cup of the apparatus 1 cc. of 10 per cent urease solution, and run into the urease solution 0.25 cc. of the 0.26 M Na_3PO_4 solution, measured by drops from the soda-lime tube (the first 2 or 3 drops are to be discarded as they have absorbed atmospheric CO_2). The urease-phosphate mixture is at once drawn down into the chamber of the apparatus, and the cock is sealed with a drop of mercury. In order to mix urease and blood, and to bring the enzyme into contact with the portions of blood solution wetting the walls of the chamber, the mercury in the latter is lowered to the bottom and then permitted to rise again. The mixture is now permitted to stand a sufficient length of time (1 minute with Squibb's urease) for the enzyme to complete its action.

Extraction and Measurement of CO_2 Formed from Blood Urea.—After the enzyme has finished its action 0.5 cc. of 1 N lactic acid is placed in the cup, and 0.25 cc. is drawn down into the apparatus, making the total volume of solution up to 7 cc. The cock is sealed with mercury, the chamber is evacuated, and is shaken 2 minutes, as in determinations of blood CO_2 . The volume of the extracted gas is reduced, ordinarily to 0.5 cc. If the blood urea content is over 75 mg. per 100 cc. (urea nitrogen over 35 mg.), however, the CO_2 pressure at 0.5 cc. volume will exceed 400 mm. With such

bloods the volume of the extracted gas is reduced only to 2 cc. The technique described by Van Slyke and Neill, on p. 533 of their paper, for reducing the volume of extracted CO_2 for pressure measurement is to be followed.

With the extracted gases at 0.5 or 2 cc. volume, the manometer reading p_1 is noted. Without releasing the vacuum, 3 or 4 drops of 5 N NaOH solution are admitted, a drop at a time, from the cup to absorb the CO_2 . The vacuum is then released and the solution is permitted to rise for a moment into the upper stem of the chamber, to wash out the alkali. The mercury in the chamber is finally lowered again until the surface of the blood solution has fallen to the 0.5 or 2 cc. mark used for the first reading, and the second manometer reading, p_2 , is taken.

A *control analysis* is carried through, in which the blood is replaced by water solution. The difference between p_1 and p_2 in the control analysis is designated as c , for the calculation. The value of c is usually 5 or 6 mm. with a gas volume of 0.5 cc., and 1 or 2 mm. with a gas volume of 2 cc. The pressure due to CO_2 from urea in the analysis is

$$P_{\text{urea}} = p_1 - p_2 - c$$

The CO_2 pressure c observed in the control analysis is due in part to a trace of CO_2 in the alkaline phosphate, in part to the trace of CO_2 present in the water used to dissolve the urease.

A total solution volume as great as 7 cc. for 1 cc. of blood is used because the 5 N sodium hydroxide can be run into it for absorption of CO_2 at the end of the analysis. If only a 3-fold dilution of whole blood were used, the 5 N alkali would cause a gummy precipitate of hemoglobin on the walls of the chamber. One would have to use 1 N alkali, which would have to be freed of air before it could be used.

Before each analysis the chamber is washed once with dilute lactic acid, as described on p. 534 of Van Slyke and Neill's paper.

Determination in 0.2 Cc. of Whole Blood or Plasma.

If the blood is drawn by skin puncture outside the laboratory a convenient procedure is to place 1 cc. of 0.02 N lactic acid in a small test-tube (6 or 7 mm. inner diameter) and draw 0.2 cc. of blood directly into a capillary pipette. The pipette is emptied into

the lactic acid, and is rinsed twice by drawing the acid up into it. The blood solution, together with a drop of caprylic-ethyl alcohol, is transferred to the chamber of the blood gas apparatus, 0.6 cc. of water, in portions of 3 or 4 drops each, being used to wash adherent drops of blood solution from the test-tube into the chamber of the blood gas apparatus

If the blood is drawn in the laboratory, the procedure may be simplified by emptying the pipette directly into the blood gas apparatus. The 1 cc. of 0.02 N lactic acid, plus 0.6 cc. of water, is then used to rinse the blood adherent to the pipette and the cup of the apparatus into the chamber

After the 0.2 cc. of blood and the acid, by either of the above procedures, have been brought into the chamber, the CO_2 is removed by extracting three times, as described above. Then 0.2 cc. of the 10 per cent urease is placed in the cup of the apparatus, together with 0.05 to 0.07 cc. of the 0.26 M Na_3PO_4 (2 drops), and the mixture is drawn down into the chamber, making the total volume of solution approximately 2 cc. The enzyme-phosphate solution is mixed with the blood by lowering the mercury to the bottom of the chamber and permitting it to rise again. The urease is allowed to act on the urea for 1 minute (or longer if a weaker enzyme necessitates it). Finally the mixture is acidified by admission of 2 or 3 drops of 1 N lactic acid, the CO_2 is extracted by 2 minutes shaking of the evacuated chamber, and the pressure is measured, p_1 , with the gas volume at 0.5 cc. The vacuum is released; 1 or 2 drops of 5 N sodium hydroxide are admitted to absorb the CO_2 , the meniscus of the solution is lowered again to the 0.5 cc. mark, and pressure p_2 is read on the manometer.

A control determination is performed in which the blood is replaced by water. The difference between p_1 and p_2 in the control analysis is designated as c . The pressure of CO_2 from urea is

$$P_{\text{urea}} = p_1 - p_2 - c$$

Before each determination the apparatus is washed twice with distilled water.

Calculation of Results of Analyses.

The factors by which the P_{urea} values obtained in the above analyses are multiplied in order to give the urea contents of urine or blood are given in Tables I, II, and III.

The factors in the tables were calculated as follows: Since 1 mol of urea yields 1 mol of CO_2 the molecular concentrations of $\text{CO}(\text{NH}_2)_2$ or urea N_2 are calculated, from the CO_2 pressures ob-

TABLE I

For Analysis of Urine.

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied.

Volume of solution extracted = 3.5 cc. Gas volume at which pressure is measured = 2 000 cc.

Temperature °C	Factors giving gm urea N per 100 cc urine		Factors giving gm urea per 100 cc urine	
	Sample = 0.1 cc urine	Sample = 0.2 cc urine	Sample = 0.1 cc urine	Sample = 0.2 cc urine
15	0 003443	0 001723	0 00738	0 003689
16	24	13	34	68
17	04	08	30	47
18	0 003386	0 001693	26	27
19	68	84	22	08
20	51	76	18	0 003589
21	33	68	14	70
22	14	59	10	51
23	0 003297	50	07	33
24	81	41	03	15
25	65	38	00	0 003497
26	50	26	0 00697	80
27	34	18	93	64
28	19	11	90	49
29	03	03	86	32
30	0 003188	0 001595	83	16
31	74	88	80	01
32	60	81	77	0.003386
33	46	74	74	71
34	32	67	71	56

served in the gas apparatus, by the same equation used for CO_2 (see Van Slyke and Sendroy (8)); viz.,

$$\text{mm CO}_2 \text{ or urea per liter} = P_{\text{urea}} \times \text{factor}$$

$$\text{Factor} = \frac{1000}{\text{cc. sample}} \times \frac{i a}{760 \times 22.26 (1 + 0.00384 t)} \left(1 + \frac{S}{A - S} \alpha' \right)$$

P is the pressure of CO_2 (from urea in the present case). a is the volume of the gas, 2.0 or 0.5 cc., at which the pressure is read. i is the empirical factor correcting for reabsorption of CO_2 , found to be 1.017 when $a = 2.0$ cc., 1.037 when $a = 0.5$ cc. t is the tem-

TABLE II

For Analysis of Folin-Wu Blood Filtrate.

Factors by Which Millimeters of Pressure from Urea CO_2 Are Multiplied.

Volume of solution extracted (S) = 5.7 cc Sample = 0.5 cc. blood.
 $\alpha' = 0.945$ α' for water

Temperature	Factors giving mg urea N per 100 cc blood		Factors giving mg urea per 100 cc blood	
	$a = 2.0$ cc	$a = 0.5$ cc	$a = 2.0$ cc	$a = 0.5$ cc
°C.				
15	0.720	0.1834	1.540	0.393
16	15	21	30	90
17	10	09	20	88
18	06	0.1798	10	85
19	01	87	00	83
20	0.697	76	1.491	80
21	93	65	82	78
22	88	54	73	76
23	84	43	64	73
24	80	33	55	71
25	76	23	47	69
26	72	13	39	67
27	68	03	31	65
28	65	0.1694	23	63
29	61	85	15	61
30	58	76	07	58
31	54	68	00	57
32	51	59	0.1393	56
33	48	51	87	54
34	45	43	80	52

perature centigrade. A is the volume of the gas apparatus chamber, usually 50 cc., S is the volume of solution extracted in the apparatus; α' is the Henry distribution coefficient, between gas phase and solution, of the gas determined.

From the millimolar factors obtained as outlined above, the factors for calculating grams of urea and urea nitrogen were ob-

TABLE III.

For Direct Analysis of Whole Blood or Plasma.

Factors by Which Millimeters of Pressure from Urea CO₂ Are Multiplied.

Temperature.	Factors to give mg urea N per 100 cc blood			Factors to give mg urea per 100 cc blood.		
	Sample = 1 cc blood		Sample = 0 2 cc blood	Sample = 1 cc blood		Sample = 0 2 cc blood
	a = 0 5 cc S = 7 0 "	a = 2 0 cc S = 7 0 "		a = 0 5 cc S = 7 0 "	a = 2 0 cc S = 7 0 "	a = 0 5 cc S = 2 0 "
°C.						
15	0 0955	0 3740	0 424	0 2048	0 0802	0 909
16	47	12	22	30	0 0796	05
17	40	0 3684	20	12	90	00
18	33	59	18	0 1998	84	0 896
19	26	34	16	84	79	92
20	19	08	14	70	73	88
21	13	0 3583	12	57	68	84
22	08	58	11	44	63	80
23	02	34	09	31	58	76
24	0 0895	10	07	17	52	73
25	89	0 3488	05	04	48	68
26	83	66	03	0 1891	43	65
27	77	45	02	78	38	61
28	72	24	00	65	34	58
29	68	04	0 398	62	30	54
30	63	0 3384	97	49	25	50
31	58	66	95	36	21	47
32	54	48	93	33	17	44
33	49	29	92	20	13	41
34	44	11	91	07	10	37

a indicates gas volume at which pressure is measured.

S indicates volume of solution from which the CO₂ is extracted in the apparatus.

tained by use of the molecular weights, 60.05 for CO(NH₂)₂ and 28.016 for N₂.

For the Folin-Wu filtrate analyses, it was found that in the mix-

ture extracted, *viz.* 1 volume of filtrate plus 0.1 volume of urease solution in 50 per cent glycerol plus 0.04 volume of 1 N lactic acid, CO_2 had a solubility only 0.945 times the solubility of CO_2 in water. Hence in calculating the factors for the Folin-Wu filtrate analyses the α' values of Table I of Van Slyke and Neill have been multiplied by 0.945. For the other analyses, in which blood or urine was diluted with water containing relatively little salt, the α' values of water were used.

Gasometric Standardization of Urease Activity.

In previous papers (5, 9) on the mode of action of urease it has been shown that the maximum activity of the enzyme is exerted at a pH of approximately 7, maintained by phosphate buffers, and a high concentration of urea. To determine the activity of urease Van Slyke and Cullen (6) caused the enzyme to act on a solution containing 0.25 M KH_2PO_4 , 0.25 M K_2HPO_4 or Na_2HPO_4 , and 1 M urea concentrations. They permitted 100 mg. of urease (1 cc. of 10 per cent solution) to act on 5 cc. of this solution at 20° for 15 minutes, then stopped the enzyme action by adding excess K_2CO_3 , and determined by aeration the amount of ammonia formed. They specified that for use in their method the ammonia should neutralize at least 8 cc. of 0.1 N acid, indicating 24 mg. of urea decomposed in 15 minutes, or 0.016 mg. of urea decomposed per minute by 1 mg. of urease.

The present Squibb's urease, made according to Cullen and Van Slyke's method, but from jack beans instead of soy beans, has from 3 to 6 times this activity: it can decompose at 20° from 0.05 to 0.10 its weight of urea per minute.

The activity is determined in the gas apparatus as follows: 2 cc. of the above phosphate-urea solution of Van Slyke and Cullen (6) at room temperature are run into the chamber of the apparatus. 1 cc. of water is placed in the cup, and 0.1 cc. of 5 per cent urease, containing 5 mg. of enzyme, is run underneath the water from a capillary pipette. The enzyme solution, followed by 0.9 cc. of water, is run into the chamber. The time is noted. The intermixture of enzyme and urea solution is quickly completed by lowering the mercury in the chamber a few centimeters and letting it rise again. After an interval, usually 5 minutes, sufficient to yield 200 to 400 mm. of CO_2 pressure, 0.5 cc. of 1 N lactic acid is

run into the chamber, the CO_2 is extracted, and the manometer reading, p_1 , is taken with the gas volume at 2.0 cc. The apparatus is washed out twice with water, and a control analysis is run without enzyme, the reading obtained being p_0 .

The number of milligrams of urea that 1 mg. of enzyme preparation can decompose in 1 minute is found by multiplying $(p_1 - p_0)$ by the factor in Table IV corresponding to the temperature, and dividing by the number of minutes the enzyme acted and the number of milligrams of enzyme present.

$$\text{Mg. urea split per minute at } 20^\circ \text{ by 1 mg. urease} = \frac{(p_1 - p_0) \times \text{factor}}{(\text{minutes action}) \times (\text{mg. urease})}$$

Example.—In standardizing the enzyme used in the present work the $p_1 - p_0$ value was 396 mm. at 22.5° , 5 mg. of the enzyme having acted 5 minutes. Inserting these values into the above formula we obtain:

$$\text{Mg. urea split per minute at } 20^\circ \text{ by 1 mg. urease} = \frac{396 \times 0.0060}{5 \times 5} = 0.095$$

Calculation of Factors in Table IV.—The $(p_1 - p_0)$ value obtained in the above standardization test, multiplied by a factor from the fourth column of Table I indicates the number of milligrams of urea decomposed. (This factor gives gm. of urea per 100 cc. of urine when 0.1 cc. is analyzed. Gm. per 100 cc., however, is the same as mg. in the 0.1 cc. sample analyzed.) However, if the temperature is above 20° , the activity of the enzyme is increased; e.g., at 30° it decomposes twice as much urea per minute as at 20° . Hence a correction factor must be introduced to bring the figure to the value it would have at 20° . Van Slyke and Cullen (5) found that between 10° and 50° the effect of temperature on urease activity is indicated by the equation:

$$\text{Log} \frac{\text{activity at } t_1^\circ}{\text{activity at } t_2^\circ} = 0.029 (t_1 - t_2)$$

where t_1 and t_2 are any two temperatures within the above range. If t_1 is 20° and t_2 is t° , the temperature of the analysis, this equation becomes

$$\text{Log} \frac{\text{activity at } 20^\circ}{\text{activity at } t} = 0.029 (20 - t)$$

which may also be expressed as

$$\frac{\text{Activity at } 20^{\circ}}{\text{Activity at } t^{\circ}} = 10^{0.029(20-t)}$$

TABLE IV

For Calculating Activity of Urease.

Factors by Which Millimeters of Pressure from Urea CO₂ Are Multiplied to Give Milligrams of Urea That Would Be Decomposed at 20°.

Temperature	Factor
°C.	
15	0 0103
16	0 0096
17	89
18	85
19	77
20	72
21	68
22	62
23	58
24	54
25	50
26	47
27	43
28	40
29	37
30	35
31	33
32	30
33	28
34	26

Hence each factor in the fourth column of Table I is multiplied by $10^{0.029(20-t)}$ in order to correct for the effect of temperature on the activity of the enzyme. The resulting combined factors are those of Table IV. They are carried out only to two places, because the velocity determinations performed as described are not sufficiently accurate to justify a third figure.

EXPERIMENTAL.

Urine Analyses.

In order to test the urine method on known solutions, Merck's urea was made up in solutions of 1, 2, and 3 per cent concentration. These were analyzed by macro-Kjeldahl determinations performed on 3 cc. samples, also by the Van Slyke-Cullen method with 0.5 cc. samples, and by the present gasometric method, with samples equivalent to 0.1 cc. of urine. The results, in Table V, represent the averages of closely agreeing triplicate estimations.

A number of urines were analyzed both by gasometric determination of the CO₂ from the urea, and by the Van Slyke-Cullen pro-

TABLE V
Analyses of Pure Urea Solutions by the Urine Urea Methods

Urea added to solution		Urea N determined.		
Urea	Urea N	Kjeldahl	Van Slyke-Cullen urease method	Present gaso- metric urease method
<i>gm. per 100 cc</i>	<i>gm. per 100 cc</i>	<i>gm. per 100 cc</i>	<i>gm. per 100 cc</i>	<i>gm. per 100 cc</i>
1 00	0 467	0 465	0 463	0 465
2 00	0 934	0 931	0 918	0 930
3 00	1 867	1 860	1 834	1 868

cedure for aerating and titrating the ammonia. The results are given in Table VI.

Blood Analyses.

Time Required for Urease Action.—To 30 cc. of horse blood, containing 11.4 mg. of urea nitrogen per 100 cc., 1 cc. of 6 per cent urea solution was added, in order to raise the urea content to that encountered in uremic blood. The calculated urea nitrogen content of the mixture was 101.4 mg. per 100 cc. Analyses were performed on 1 cc. samples by the procedure described above for whole blood. The period permitted for action of the urease, however, was varied by making the final addition of lactic acid at varying intervals after the enzyme and blood had been mixed in the gas apparatus. The urease was that described above in connection with the standardization: 100 mg. (the amount employed) could

TABLE VI

Comparison of Urea Determinations in Urine by the Van Slyke-Cullen Ammonia Titration Method and the Present Gasometric CO₂ Method

Urine No	Gasometric measurement of CO ₂				Van Slyke-Cullen titration of NH ₃		
	Urine volume in sample used for determination	Observed pressure at 2 cc volume of CO ₂ from urea	Temperature	Urea N per 100 cc urine	0.02 N HCl required in titration	Urine volume used for analysis	Urea N per 100 cc urine
	cc	mm	°C	gm	cc	cc	gm
1	0 1	276 4	19 0	0 931	16 52	0 5	0 925
	0 1	275 6	19 0	0 928	16 56	0 5	0 927
2	0 1	283 4	19 0	0 955	16 82	0 5	0 942
	0 1	283 8	19 0	0 956	16 80	0 5	0 941
3	0 2	274 2	19 7	0 460	8 11	0 5	0 459
	0 2	271 9	19 7	0 456	8 16	0 5	0 464
4	0 2	255 4	19 9	0 428	7 49	0 5	0 419
	0 2	256 6	19 9	0 428	7 58	0 5	0 429
5	0 2	88 8	19 4	0 149	2 58	0 5	0 145
	0 2	90 5	19 5	0 152	2 53	0 5	0 142
6	0 2	100 1	19 9	0 168	2 94	0 5	0 165
	0 2	101 2	20 4	0 169	2 91	0 5	0 163
7	0 1	104 8	22 0	0 347			
	0 1	104 8	22 0	0 347			
	0 2	207 7	20 5	0 347			
	0 2	207 0	20 5	0 346	6 16	0 5	3 45
	0 2	207 7	21 5	0 346	6 20	0 5	3 47
8	0 1	117 8	21 8	0 391			
	0 2	235 6	20 8	0 393			
	0 2	235 2	20 5	0 393	6 96	0 5	3 90
	0 2	236 7	21 5	0 394	6 96	0 5	3 90
9	0 1	64 8	22 5	0 214			
	0.2	126 6	21 0	0 211			
	0 2	126 2	20 5	0 211	3 77	0 5	2 11
	0 2	126 4	21 5	0 210	3 83	0 5	2.15

decompose a maximum of 9.5 mg. of urea in 1 minute at 20°. The action on the blood was so rapid that it was complete within a half minute, which was about the time required, after mixing the blood and enzyme, to measure out and add the acid. The results are given in Table VII, together with those by the Van Slyke-Cullen aeration-titration procedure.

Analyses of Standard Urea Solutions.—By dilution of a stock 1 per cent urea solution, 0.200, 0.100, and 0.050 per cent urea solutions were prepared. They were analyzed by the procedures described above for blood urea, and also by the Van Slyke-Cullen

TABLE VII
Time Required for Action of 1 Cc of 10 Per-Cent Urease on 1 Cc of Uremic Blood

Gasometric determination on 1 cc blood					Van Slyke-Cullen titration of NH_3 from 3 cc blood	
Period of digestion with urease	Pressure of CO_2 from urea, measured at 2 cc volume	Temperature	Factor	Urea N per 100 cc blood	0.01 N acid neutralized	Urea N per 100 cc blood
<i>min</i>	<i>mm</i>	$^{\circ}\text{C}$		<i>mg</i>	<i>cc</i>	<i>mg</i>
0 5	278 8	19 0	0 3634	101 4	21 89 21 92	102 1 102 3
1 0	275 8	18 5	0 3646	100 6		
2 0	276 9	18 6	0 3644	100 9		
4 0	277 5	19 0	0 3634	100 9		

procedure. For greater accuracy, the latter was performed on 5 cc. samples instead of the 2 or 3 cc. portions usually employed in routine blood analyses. The results are given in Table VIII.

Analyses of Nephritic Bloods.—In Tables IX and X are given data from a number of nephritic bloods, showing varying degrees of urea retention, with the results of urea determinations by the gasometric methods described above. Parallel results by the Van Slyke-Cullen method are also given. The agreement between the results obtained by NH_3 and CO_2 determination respectively affords evidence of the specificity of the enzyme for urea, among the blood constituents.

TABLE VIII.

Comparison of Results of Analyses of Standard Urea Solutions by Gasometric Blood Urea Methods and by Van Slyke-Cullen Method.

Solution	Method.	Gasometric measurement of CO ₂						Titration of NH ₃ .	
		Volume of solution equivalent to sample	Gas volume at which CO ₂ pressure was measured	Pressure of CO ₂ from urea	Temperature	Factor.	Urea N per 100 cc	0.01 N HCl neutralized by NH ₃ from 5 cc. solution	Urea N per 100 cc.
		cc	cc	mm	°C		mg	cc.	mg.
Urea solution 200 mg urea, 93.3 mg N, per 100 cc	Whole blood.	1 000	2 000	257 0	19 0 0	3634	93 5	32 78	91 8
		1 000	2 000	257 1	19 7 0	3616	93 0	32 55	91 2
	Filtrate A.	0 500	2 000	135 1	21 1 0	693	93 6		
		0 500	2 000	134 1	21 1 0	693	92 9		
		0 500	2 000	134 0	21 1 0	693	92 9		
	Filtrate B	0 500	2 000	134 2	21 0 0	693	93 0		
		0 500	2 000	133 8	21 0 0	693	92 8		
	Whole blood	1 000	2 000	127 6	20 0 0	3608	46 1	16 34	45 8
		1 000	2 000	128 0	20 0 0	3608	46 2	16 42	46 0
		1 000	2 000	127 5	20 0 0	3608	46 0		
	Filtrate A.	0 500	2 000	69 0	21 2 0	692	47 1		
		0 500	2 000	68 3	21 2 0	692	46 5		
		0 500	2 000	68 5	21 2 0	692	46 7		
		0 500	0 500	266 7	21 2 0	1763	47 0		
		0 500	0 500	261.5	21 2 0	1763	46 1		
		0 500	0 500	265 0	21 2 0	1763	46 7		
	Filtrate B	0 500	0 500	262 4	21 0 0	1765	46 3		
		0 500	0 500	263 8	21 0 0	1765	46 6		
Urea solution 50 mg urea, 23.3 mg N, per 100 cc	Whole blood.	1 000	2 000	64 7	20 0 0	3608	23 3	8 09	22 7
		1 000	2 000	64 4	20 0 0	3608	23 2	8 09	22 7
		1 000	0 500	249 7	20 0 0	0919	22 9		
		1 000	0 500	249 6	20 0 0	0919	22 9		
	Filtrate A	0 500	0 500	133 3	21 2 0	1763	23 5		
		0 500	0 500	131 5	21 2 0	1763	23 2		
		0 500	0 500	131 7	21 2 0	1763	23 2		
	Filtrate B.	0 500	0 500	131 8	21 0 0	1765	23 3		
		0.500	0 500	133 0	21 0 0	1765	23 5		

CORRECTIONS.

On pages 721 and 722, Vol. lxxiii, No. 2, June, 1927, Table 1X, in the heading of the next to the last column, read *0.01 N HCl* for *1.025 N HCl*.

TABLE IX.

Comparison of Blood Urea Determinations by Gasometric Methods and by Van Slyke-Cullen Titration Method.

Blood No	Gasometric measurement of CO ₂ .							Titration of NH ₃ .	
	Method.	Volume of blood equivalent to sample	Gas volume at which CO ₂ pressure was measured	Pressure of CO ₂ from urea	Temperature	Factor	Urea N per 100 cc blood	1 025 N HCl neutralized by NH ₃ from 2 cc blood.	Urea N per 100 cc blood.
		cc	cc	mm	°C		mg	cc	mg
1	Whole blood	1 000	0 500	283 9	24 5	0 0892	25 3	3 49	24 4
	" "	1 000	0 500	280 3	23 1	0 0902	25 3	3 60	25 2
2	Whole blood	1 000	0 500	69 7	23 5	0 0898	6 26	0 88	6 2
	" "	1 000	0 500	70 8	23 7	0 0897	6 35	0 98	6 9
3	Whole blood	1 000	0 500	379 7	24 0	0 0895	34 0	4 85	34 0
	" "	1 000	0 500	376 7	24 0	0 0895	33 7	4 74	33 2
4	Whole blood	1 000	2 000	147 4	24 5	0 3499	51 6	7 36	51 6
	" "	1 000	2 000	147 4	24 5	0 3499	51 6	7 42	51 9
5	Filtrate A	0 500	0 500	150 8	23 5	0 1738	26 2		
	" "	0 500	0 500	151 8	23 5	0 1738	26 3		
	Whole blood	1 000	0 500	301 4	23 0	0 0902	27 2	3 80	26 6
	" "	1 000	0 500	301 2	23 0	0 0902	27 2	3 81	26 7
6	Filtrate A.	0 500	0 500	146 4	23 5	0 1738	25 4		
	" "	0 500	0 500	146 3	23 5	0 1738	25 4		
	Whole blood	1 000	0 500	291 0	24 0	0 0895	26 0	3 62	25 3
	" "	1 000	0 500	290 3	24 0	0 0895	26 0	3 64	25 5
7	Filtrate A.	0 500	0 500	75 1	22 5	0 1748	13 1		
	" "	0 500	0 500	74 6	22 5	0 1748	13 0		
	Whole blood	1 000	0 500	139 2	23 0	0 0902	12 55	1 76	12 3
	" "	1 000	0 500	138 4	23 0	0 0902	12 48	1 76	12 3

TABLE IX—*Concluded*

Blood No	Gasometric measurement of CO ₂							Titration of NH ₃	
	Method	Volume of blood equivalent to sample	Gas volume at which CO ₂ pressure was measured	Pressure of CO ₂ from urea	Temperature	Factor	Urea N per 100 cc blood	1 025 N HCl neutralized by NH ₃ from 2 cc blood	Urea N per 100 cc blood
		cc	cc	mm	°C		mg	cc	mg
8	Filtrate A	0 500	2 000	70 2	22 3	0 687	48 9		
	“ “	0 500	2 000	69 7	22 3	0 687	48 6		
	Whole blood	1 000	2 000	135 8	22 8	0 3530	48 0	6 76	47 4
	“ “	1 000	2 000	135 7	22 8	0 3530	48 0	6 76	47 4
9	Filtrate A	0 500	0 500	127 7	23 1	0 1742	22 2		
	“ “	0 500	0 500	127 4	23 1	0 1742	22 2		
	Whole blood	1 000	0 500	251 0	24 0	0 0895	22 5	3 19	22 3
	“ “	1 000	0 500	250 0	24 0	0 0895	22 4	3 26	22 8

TABLE X

*Urea Content of Nephritic Blood by Microgasometric Method.
Blood Sample of 0.2 Cc.*

Blood No	Pressure of CO ₂ at 0.5 cc volume	Temperature	Factor	Urea N per 100 cc blood	Urea N per 100 cc blood by Van Slyke-Cullen method
	mm	°C		mg	mg
1	94 9	23 0	0 409	38 8	39 0
2	91 1	23 0	0 409	37 3	36 9
	91 1	23 0	0 409	37 3	
3	99 2	22 3	0 410	40 7	40 7
4	91 6	23 0	0 409	37 5	37 5
5	85 6	22 5	0 410	35 1	35 8
6	104 2	23 0	0 409	42 6	43 4
	104 2	23 0	0 409	42 6	
7	103 9	22 5	0 410	42 6	41 7
	103 0	22 5	0 410	42 2	

SUMMARY.

Methods are described for rapid determination of urea in blood and urine by measuring in the manometric blood gas apparatus the CO_2 of the ammonium carbonate formed by action of urease. The blood urea may be determined in either whole blood, serum, or Folin-Wu filtrate. Micro determinations may be performed with 0.2 cc. of blood.

The methods here presented were developed with the technical assistance of John Plazin.

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THE EFFECT OF INSULIN ON THE RESPIRATORY EXCHANGE OF FED AND FASTING RABBITS.*

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Notwithstanding the very great number of observations which have been reported bearing on the changes produced in the respiratory metabolism of animals after injection with insulin, which have been reviewed elsewhere (Macleod, 1926), it was considered advisable to repeat them again paying particular regard to the nutritional condition of the animal and to the avoidance of hypoglycemic convulsions. As a result of their researches on eviscerated animals, Best, Dale, Hoet, and Marks (1926) have concluded that the increased disappearance of carbohydrate which insulin brings about in the non-diabetic animal can be accounted for by the overactivity of those same processes which are subnormal in diabetes, namely oxidation of carbohydrate, and storage of glycogen, and by inhibition of the process of gluconeogenesis. This view makes it unnecessary to assume that any of the disappearing carbohydrate becomes converted into non-carbohydrate substances, but before accepting the conclusion of these workers as final it seemed advisable to repeat the observations on intact animals. In so far as glycogen formation is concerned this has been done by Barbour, Chaikoff, Macleod, and Orr (1927) with results which show that it is only after the prolonged action of very large amounts of insulin in animals in which absorption of carbohydrate is at a maximum that the muscles lay on an excess of glycogen, which, moreover, about corresponds to a deficit in the amount of glycogen meanwhile stored in the liver. In the present

* The expenses of this investigation were in part defrayed by a grant from the Carnegie Corporation.

investigations the possibility of increasing the rate of glycogen formation and of depressing that of gluconeogenesis has been reduced to a minimum by previous rich feeding with carbohydrate, leaving increased oxidation as the only one of the above three processes that could be affected by insulin.

The experiments are arranged in three groups. In the first group (Table I) rabbits which had been fed for some days previously with carrots and oats were used, and this food was also placed along with the animals in the respiratory cabinet. Under these conditions it is to be assumed that the glycogen stores are filled to their capacity, so that metabolism will be almost completely restricted to preformed carbohydrate and gluconeogenesis will be reduced to a minimum.

In the second group (Table II) fasting animals were used in which therefore the energy requirements were supplied mainly by fat and protein. Assuming that these foodstuffs must be converted into carbohydrate before their energy can be set free gluconeogenesis will be at its maximum.

In the third group (Table III) the animals were in varying states of nutrition.

In the experiments of Groups I and II the metabolism of each rabbit was measured from hour to hour, on 1 day without insulin and on the next day with insulin. This is a much more reliable method of comparison than that adopted in the experiments of Group III in which the normal metabolism was measured for two periods of 1 hour each after which insulin was injected and the metabolism again measured for three or four hourly periods. The sources of inaccuracy in this last mentioned method depend on the fact that greater variability is liable to occur between the results of the first 2 (normal) hours than between those of later hours, partly because the animal is apt to move about more and partly because of the unavoidable accumulation of a certain percentage of CO_2 in the air of the chamber. To minimize this error the rule in the present investigation has been to allow the animal to be in the chamber (with the absorbers in circuit) for about half an hour before commencing the actual observations. Sometimes, however, this period is insufficient to insure a stable condition of the air with regard to percentage of CO_2 and temperature so that the results of the 1st hour are less dependable than those of later

hours. This source of error does not appreciably enter into the more prolonged observations of Groups I and II, since the percentage of CO_2 in the air of the cabinet remains reasonably constant after the 1st hour, as we have repeatedly observed by analysis. It may be stated, in parenthesis, that we have considered 0.15 as the highest percentage to which CO_2 may be allowed to rise. Since the capacity of the chamber is 160 liters this equals 240 cc. of CO_2 . Since at least 1000 cc. of CO_2 are produced per hour by a rabbit of average size, or 6000 cc. in 6 hours, a total maximal error of 4 per cent is possible in the CO_2 and therefore also in O_2 . This would be serious were absolute values being striven for, but we have disregarded it in the present investigations since we have found the accumulation of CO_2 to be constant when the blower is running at a certain speed, which has always been the case when the results of one period were being compared with those of another.

Alcohol and ether tests were frequently run in the chamber and almost invariably the observed respiratory quotient corresponded exactly with the theoretical. The total volume of oxygen consumed also corresponded closely with the theoretical, the possible source of error due to accumulation of CO_2 in the air of the chamber being in this case eliminated by leaving the blower and absorbers in operation for some time after extinguishing the flame. That exact correspondence was not always attained between the observed and the theoretical O_2 in these tests was mainly due to the sudden rise in temperature when the flame was lighted. Further details of the respiratory cabinet used and of the results of the ether and alcohol tests will be published later.

The numbered columns in Tables I and II give the oxygen consumption per kilo for each hour from about 8 a.m. to 2 p.m., along with the corresponding respiratory quotients. From the sum of the oxygen values and the average of the R.Q.'s the calorie consumption and the carbohydrate utilization for the 6 hours are calculated (end columns). In the majority of the observations this is done without paying any regard to the nitrogen excretion, since this has been found by Macleod and Allan (1923) and by Sokhey and Allan (1924) to undergo no significant change in urine collected over a period of 24 hours as a result of subconvulsive doses of insulin. To have collected urine by catheter over shorter

TABLE I.
O₂ per Kilo per Hour and Corresponding R. Q

Hour		1		2		3		4		5		6		Averages		Calories per kilo	Carbohydrate per kilo for period.	Difference	
Date.	Rabbit No	Weight	Range of temperature of chamber.	O ₂	R	Q	O ₂	R	Q	O ₂	R	Q	O ₂	R	Q		gm	gm	
		gm	°C	cc			cc			cc			cc						
Sept. 7	XIII	3750	20 6 -22 45	500 1 05	638 1 04	628 1 01	628 1 01	620 1 04	663 1 03	646 1 01	615 1 03	18 6 4	973						
" 8	XIII	3750	20 4 -22 6	557 1 05	616 1 04	573 1 03	616 1 08	540 1 04	557 0 984	577 1 04	17 45 4	666	-0 31						Insulin 10 units. Blood sugar not determined *
Sept. 9	XIV	3600	21 45-22 85	5160 959	540 1 03	570 1 02	596 0 981	550 1 01	585 1 09	560 1 01	16 98 4	540							Insulin 10 units. Blood sugar not determined.
" 10	XIV	3600	19 95-21 83	5890 88	634 1 05	657 1 07	637 1 03	581 1 01	697 0 99	603 1 01	19 06 5	096	+0 56						
Oct. 25	I	3360	19 8 -22 55	717 1 01	709 1 03	775 1 01	690 0 991	720 1 04	718 1 02	723 1 02	21 90 5	856							Insulin 10 units. Blood sugar in 3½ hrs 0 120 per cent.
" 26	I	3250	19 4 -23 03	7590 998	804 0 981	751 1 00	7320 939	755 0 987	783 1 02	764 0 987	23 14 5	867	+0 011						
Oct. 27	II	2600	20 85-23 9	7450 958	715 0 983	700 1 04	740 0 961	799 0 989	683	730 1 00	22 08 5	839							Insulin 10 units. Blood sugar in 3½ hrs. 0 08 per cent.
" 28	II	2650	22.15-24 63	672 1 00	772 1 02	752 1 05	716 1 01	665 1 03	682	710 1 03	21 49 5	746	-0 093						
Dec. 2	VI*	2900	22.10-23 80	700 1 10	780 0 905	694 0 878	790 0 985	725 0 984		738 0 97	18.62	4.44							Insulin 10 units. Blood sugar not determined.
" 3	VI	2880	21.40-23.25	543 1.13	726 1.01	790 1 01	856 0 955	781 1.04		740 1 05	18.65	4.80	+0.100						

periods was not considered advisable, because of the necessary disturbance of the animal. In those cases in which urine was collected over the 24 hours (as in Rabbit VI) the results showed that the number of calories contributed by protein was less than 10 per cent of the total. Thus, in the observations of December 2 and 3 the urine of 24 hours contained 0.79 gm. of N. This corresponds to 0.033 gm. per hour or $0.033 \times 26.5 = 0.875$ calories per hour, or $\frac{0.875 \times 5}{2.9} = 1.47$ calories per kilo for the observation period of 5 hours, which is 7.8 per cent of the total calorie expenditure during this period.

In Tables I and II the observations on each animal are arranged in pairs, the top line in each pair giving the results on the normal animal and the lower line, those on the next day when 10 units of insulin were injected. The most significant figures are those of the last two columns which give the gm. of carbohydrate metabolized during each 6 hour period.

Fed Animals with R.Q. of 100 (Table I).—The amount of carbohydrate used per kilo of body weight by each animal on the days when no insulin was given varied from 4.54 to 5.86 gm., the average for the four animals in which the period of observation was 6 hours being 5.302 gm. On the insulin days the values varied from 4.66 to 5.85 gm. with an average of 5.343 gm. This indicates that insulin did not cause any significant increase in the metabolism of carbohydrate, a conclusion which, in general, is also sustained by comparing the results obtained on the normal and insulin days of each animal. The only exception is seen in Rabbit XIV in which 0.56 gm. more carbohydrate was consumed on the insulin day, but this probably falls within the range of variation for the same animal from day to day when the temperature conditions are not quite constant as in this experiment. It is significant that in the three observations of the present series in which the temperature in the cabinet remained most constant, there was practically no difference between the carbohydrate utilization of the control and insulin days.

If in place of averages we regard the respiratory exchange for each of the hour periods of the observation (cf. numbered columns of table) it can be seen that the respiratory quotients remain unchanged from hour to hour but that there is a certain degree of

change in the O_2 consumption. In most of the observations this rises somewhat in the 2nd as compared with the 1st hour. Sometimes (as in Rabbits XIII and VI) the value for the 1st hour is very decidedly below that of the 2nd or subsequent hours, which, as has already been explained, may be due to an initial lag in the removal of CO_2 from the chamber by the ventilating current (p 726). During the subsequent periods a certain degree of variability is to be noted in the O_2 consumption. This is unavoidable since the animals were sometimes perfectly quiet and at other times were moving about the chamber and feeding. We realized when we undertook the experiments that this factor would make it impossible to obtain perfectly uniform values; but we considered, and the results justify our opinion, that the average degree of activity over a period of 6 hours would be practically the same for different animals. Bearing these facts in mind it may be permissible to compare the average O_2 consumption for each hour, as is done in the following table:

Hour	1	2	3	4	5	6
Control	635	676	673	687	691	658
Insulin.	624	710	704	711	664	679

These figures are also shown in curves A of Fig. 1.

The total of O_2 used in all periods for the control animals is 4020 cc. and 4092 cc. for the insulin-treated ones, the difference, *viz.* 72 cc., representing 0.0933 gm. of carbohydrate which agrees with the value as calculated from the figures of the last column of Table I, *viz.* 0.096 gm.

The blood sugar was not as a rule determined during these experiments to avoid possible disturbance of the animal. In controlled rabbits not placed in the respiratory cabinet the blood sugar was examined at frequent intervals following injection of insulin. Since such results have not previously been published we give typical ones here.

Effect of 10 Units of Insulin on Blood Sugar of Well Fed and Still Feeding Rabbits.

Well Fed Rabbits.—Feb. 21. Animals fed on oats and carrots for 3 days previous to experiment. On day of experiment, food removed from cage.

Black rabbit, weight 3700 gm		White rabbit, weight 2220 gm.	
Insulin		Control.	
Time.	Blood sugar.	Time.	Blood sugar
	<i>per cent</i>		<i>per cent</i>
10 14	0 139	10 20	0 160
10 25	10 units insulin.	11 20	0 173
10 55	0 068	12 20	0 177
11 25	0 082	1 20	0 180
12 25	0 070	2 20	0 157
1 25	0 056	3 20	0 167
2 25	0 106	4 40	0 136
3 25	0 142		
5 00	0 143		

No signs of convulsions.

Still Feeding Rabbits.—Feb. 8. Animals fed previous to and after injection of insulin. Food consisted of oats and carrots.

Black rabbit, weight 3880 gm.		White rabbit, weight 2400 gm.	
Insulin.		Control.	
Time.	Blood sugar.	Time.	Blood sugar
	<i>per cent</i>		<i>per cent</i>
10 50	0 161	11 08	0 135
10 56	10 units insulin.	2 38	0 176
11 26	0 098	4 08	0 146
11 56	0 085	6 45	0 151
12 56	0 102		
1 56	0 135		
2 56	0 097		
3 56	0 149		
4 56	0 149		
6 30	0 171		

No signs of convulsions.

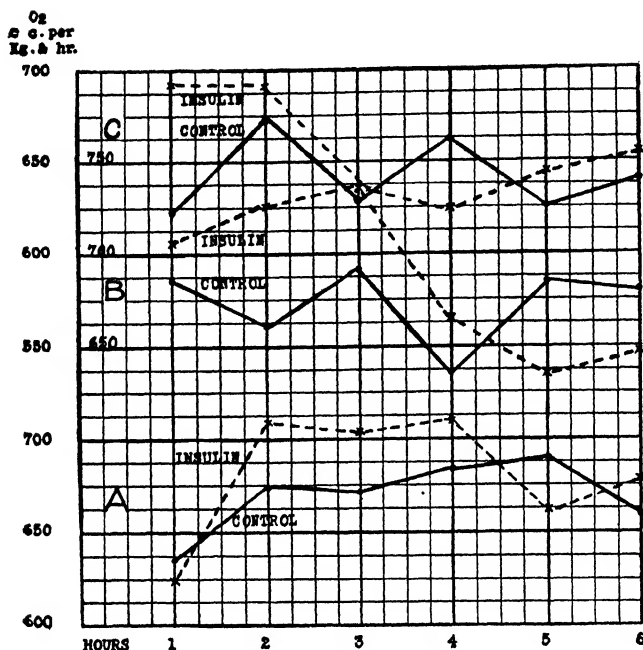


FIG. 1. Curves of averages of O_2 consumption and RQ from hour to hour. A, O_2 consumption in fed rabbits B, O_2 consumption in fasting rabbits. C, R.Q. in fasting rabbits (R.Q. in fed rabbits remained at unity throughout).

It can be seen that 10 units of insulin lower the blood sugar in previously fed rabbits, which have no access to food following the injection, to a much greater extent than in previously fed rabbits which have free access to food. Under the latter conditions this dose of insulin reduces the blood sugar to about 0.085 per cent as compared with about 0.160 per cent, at which level it stands under rich feeding conditions. The blood sugar level fluctuates considerably in still feeding animals, especially after insulin, but the hypoglycemic influence is still evident 4 hours after injection.

The conclusion is drawn that insulin causes no significant change in the respiratory metabolism when injected into rabbits in which, on account of rich carbohydrate feeding, the respiratory quotient is unity.

TABLE II
C.c. of O₂ per Kilo per Hour and Corresponding R. Q.

Hour				1		2		3		4		5		6		Averages.		Calories per kilo for period.	Carbohydrate per kilo for period.	Difference.	
	Date	Rabbit No.	Weight.	Range of tempera- ture of chamber. °C.	O ₂	R. Q.	O ₂	R. Q.	O ₂	R. Q.	O ₂	R. Q.	O ₂	R. Q.	O ₂	R. Q.	O ₂	R. Q.				O ₂
	Oct. 18	I	3350	19 9 -22 5	5020	757	536	0 815	5740	741	540	0 788	5420	744	5460	730	541	0 760	15 420	0 759		
	" 19	I	2900	19 3 -22 5	6470	779	637	0 812	6550	749	598	0 730	5680	771	5370	818	607	0 776	17 391	0 095	+0 334	
	Nov. 17	I	2650	22 0 -23 7	5560	708	552	0 751	5500	718	495	0 719	5110	732	5470	697	535	0 719	15 120	0 162		
	" 18	I	2625	21 8 -23 6	5960	807	564	0 728	6190	689	565	0 719	4600	771	5490	731	558	0 740	15 860	0 492	+0 33	
	Oct. 20	II	2600	20 0 -23 2	4620	772	575	0 745	5880	757	481	(0 890)	5380	741	6120	780	539	0 778	15 420	0 994		
	" 21	II	2550	19 6 -22 7	5390	778	617	0 864	5530	834	496	0 759	4710	717	4360	739	518	0 787	14 881	0 082	+0 088	
	Nov. 19	II	2250	21 55-23 9	7760	678	769	(0 64)	7240	680	685	0 727	7560	747	6560	769	719*	0 720	16 900	0 217		
	" 22	II	2200	20 9 -22 9	6470	829	670	0 814	6840	754			6150	767	6710	772	657	0 778	15 771	0 060	+0 843	
	Nov. 23	III†	3000	21 8 -23 9	6230	705	600	0 745	5230	755	490	0 779	5690	682	5480	730	559	0 716	16 1	0 357		
	" 24	III†	2900	22 8 -23 6	6180	762	641	0 744	6800	670	609	0 705	5690	709	5600	716	613	0 719	17 290	0 542	+0 185	

* Omitting 2nd hour.

† Corrected for N.

Observations on Fasting Rabbits (Table II).—The respiratory metabolism of two of the rabbits used in the observations of Table I (Nos. I and II) was also observed, by the same technique, after the animals had been fasting for several days, and the results are shown in Table II. In these observations no measurement was made of the excretion of nitrogen, but the table contains an observation on another rabbit in which this was done (Rabbit III). In this last animal 1.152 gm. of N were excreted on the control day and 0.912 gm. on the day on which insulin was given. This is a decidedly greater excretion than that observed in Rabbit VI (Table I), which was of the same weight. It equals 0.048 gm. of N per hour, or $26.5 \times 0.048 = 1.272$ calories per hour, or $\frac{1.272 \times 6}{3} = 2.544$ calories per kilo for the period of 6 hours, which is 15.7 per cent of the total calorie expenditure, as compared with 7.8 per cent for the fed animal. This difference, no doubt, is due to the protein-sparing influence of carbohydrate acting in the fed animal. On the day when insulin was given to this animal (Rabbit III) the average hourly excretion of nitrogen was 0.038 gm., or $26.5 \times 0.038 = 1.007$ calories per hour, or $\frac{1.007 \times 6}{3} = 2.014$ calories per kilo, which is 11.5 per cent of the total calorie expenditure. The difference between the protein calories for the 2 days is $2.544 - 2.014 = 0.530$ calories, corresponding to 0.14 gm. of carbohydrate, and it is such as to indicate that no serious error is introduced in the observations by omitting consideration of the protein metabolism. When allowance is made for protein, the respiratory quotient (as in Rabbit III) falls close to that given by Lusk as indicative of exclusive fat metabolism. The quotients of the other animals have not been corrected for protein.

The uncorrected quotients for the 6 hour periods of observation varied between 0.719 and 0.778, with an average of 0.744. On the days on which insulin was given the uncorrected quotient varied between 0.740 and 0.787, with an average of 0.770. There is therefore evidence that insulin caused the quotient to rise, but not sufficiently to indicate any very significant increase in the relative amount of carbohydrate undergoing metabolism.

Turning to the metabolism as a whole, we note that the calorie expenditure per kilo of body weight for the 6 hour period was de-

cidedly less in the fasted, as compared with the fed animals. This may have been due in part to greater muscular activity in the case of the fed animals, but is mainly dependent on the specific dynamic action of the food. The averages are as follows:

	Fed animals.	Fasting animals
Calories per kilo for 6 hrs.19 63	15 79

It will also be noted that the values from hour to hour are much more constant than are those of the fed animals, the only exception being the result on Rabbit II (November 19) which was unusually high. It is recorded in the notes that this animal throughout the observation moved about the cage much more than is usually the case for fasting animals.

The following figures give the average of the calorie expenditure for the control and the insulin days:

Control days, 15 79 calories per kilo for period of 6 hrs.
Insulin " 16 24 " " " " " " " 6 "

The slightly greater expenditure on the insulin days here demonstrated (less than 3 per cent) is of little significance in view of the varying degree of muscular activity of the animals. When the comparisons are made for each rabbit it can be seen that insulin caused slight increase in calorie expenditure in three and a decrease in two of the five observations.

When the actual consumption of carbohydrate is calculated, by the use of the Zuntz-Schumburg-Lusk table, all the observations reveal an increase as a result of insulin, this increase being dependent on the somewhat higher respiratory quotients. Taking averages, only about 0.5 gm. of carbohydrate per kilo was used by each animal in the fasting condition without insulin instead of 5.3 gm. Under the influence of insulin this average rose to 0.85 gm. so that the hormone stimulated the combustion of about 0.35 gm. of extra carbohydrate, the actual increases in the different observations varying between 0.09 and 0.84 gm. Since 0.35 gm. of carbohydrate corresponds to 1.31 calories about 8 per cent more of the total calorie expenditure came from this source after insulin.

Since the changes in R.Q. from hour to hour are not marked it may be permissible to compare the average hourly O_2 consumption

for all the observations, as has been done in the following tabulation and in curves *B* of Fig. 1:

Hour	1	2	3	4	5	6
Control.	584	561	592	538	583	582
Insulin.	609	626	638	567	536	550

It is clear that the metabolism is increased by insulin during the 3 or 4 hours following its injection, this being followed by a slight depression during the last 2 or 3 hours.

Taking these results as a whole, we conclude that according to alterations in the respiratory exchange insulin in dosage short of that capable of causing convulsions distinctly stimulates carbohydrate combustion in previously fasted rabbits.

In the third group of experiments the rabbit was placed for 2 hours in the respiratory chamber before insulin was injected, and the average metabolism of this period was compared with that of a period of 4 or 5 hours following the injection. The results are shown in Table III. The most significant figures are those of the last columns. They give the gm. of carbohydrate metabolized per kilo of body weight and per hour, as calculated from the figures of the immediately preceding columns (average calories per kilo and hour) by the use of the Zuntz-Schumburg-Lusk tables. The O_2 values and R.Q. for each hour are given in the numbered columns.

In all of the experiments, except that of Rabbit V (in which the animal was fasting), the animals were previously fed on oats, carrots, and sugar which were also placed with the animal in the respiratory cabinet. During the experiments of July when the room temperature was excessively high (about 90°F.) the animals did not feed well, often abstaining from food entirely while in the cabinet. The respiratory quotients, as well as the calorie expenditure, therefore, stand at varying levels in the different experiments, and it will be convenient to reclassify the results according to the quotients. This has been done in Table IV.

The extra glucose metabolized as a result of insulin varies between 0.045 and 0.225 gm. per hour and kilo of body weight and no parallelism is evident between this amount and the magnitude

TABLE III.

Date	Rab- bit No *	Weight. gm.	Range of temperature of chamber °C	Hrs before insulin.						Hrs. after insulin										Averages (per kilo and hr.)						Differ- ence.																
				1			2			3			4			5			Before insulin		After insulin		Calorie.		Carbohy- drate.																	
				O ₁	R	Q	O ₁	R	Q	O ₁	R	Q	O ₁	R	Q	O ₁	R	Q	O ₁	R	Q	O ₁	R	Q	O ₁		R	Q	Before	After.	Before	After.										
				cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc		cc	gm.	gm.	gm.	gm.											
July 19	VII	2730	21.65-25.3	590	0	977	839	0	973	688	0	976	731	0	2	787	1	02	796	1	02	665	1	02	714	0	975	733	1	00	3	57	3	69	0	870	0	987	+0.117			
"	27	VII	2700	21.8-24.8	627	1	01	671	1	00	691	0	99	693	1	01	701	0	976	648	1	01	†			649	1	05	683	0	997	3	27	3	44	0	874	0	919	+0.045		
"	20	VIII	2600	22.9-25.7	532	0	775	604	0	700	516	0	822	683	0	831	618	0	778	597	0	836	612			829	568	0	735	605	0	819	2	68	2	92	0	071	0	296	+0.225	
"	22	X	2550	26.2-27.8	784	0	779	718	0	841	597	0	904	777	0	807	783	0	857	833	0	800				752	0	809	747	0	844	3	62	3	62	0	338	0	440	+0.102		
"	21	IX	2430	24.8-27.6	645	0	760	570	0	912	492	1	00	741	0	814	638	1	09	786	0	885				607	0	831	664	0	937	2	94	3	28	0	330	0	692	+0.362		
"	28	IX	2500	23.0-23.75	542	0	923	600	0	869	591	0	844	678	0	938	679	0	940	722	0	888				572	0	892	667	0	906	2	81	3	29	0	476	0	580	+0.104		
Nov. 29	VI	2500	20.45-22.8	See average.										6030	755	673	0	807	655	0	755	567	0	700				657	0	726	624	0	754	3	09	2	95	0	04	0	11	+0.07
Dec. 1	VI	2450	20.9-22.7	" "										5980	70	591	0	72	588	0	73							488	0	702	592	0	720	2	29	2	78	0	00	0	08	+0.08

* All fed except Rabbit V which was fasted.

† Severe convulsions.

‡ Nitrogen for 24 hours: Nov. 29, 0.71 gm.; Dec. 1, 0.90 gm.

TABLE IV

	Initial R Q	Extra carbo- hydrate metabolized per kilo and hr as a result of insulin		Calores per kilo and hr before insulin	Remarks	Insu- lin
		gm	calo- ries			units
Rabbit VII.						
July 27	1 05	0 045	0 166	3 27	Weather 22.5°C. Convul- sions	20
" 19	0 975	0 117	0 437	3 66	Weather 22 5°C.	10
" 28	0 89	0 104	0 385	2 81	" 21 2 " Mild con- vulsions	15
" 22	0 81	0 102	0 377	3 62	Weather 26.25°C. (very sultry).	10
" 20	0 735	0 225	0 832	2 68	Weather 22 2°C. (hot and close)	10
Nov 29	0 725	0 070	0 260	2 95	Weather 17 0°C. Blood sugar 3½ hrs 0 097 per cent.	3
Rabbit V.						
Dec 1	0 70	0 080	0 296	2 78	Blood sugar 3½ hrs. 0 062 per cent	3
Average .				3 11		

of the R.Q. during the preliminary periods. The average of the values is 0.105 gm. or 0.270 gm. for a rabbit of average weight (2.55 kilos). Since we do not know, either, how much glycogen may have been present in the liver and muscles to start with, or how this may have been influenced by insulin, it is possible under the conditions of these observations that increased combustion of carbohydrate may in itself account for the fall in blood sugar. In the only experiment in which R.Q. was at unity to start with and in which therefore any stimulation of the oxidation of carbohydrates due to insulin could be revealed solely by an increase in O₂ consumption, a very small increase occurred.

DISCUSSION.

The results of the foregoing experiments confirm those originally obtained in this laboratory to the effect that the injection of sub-convulsive doses of insulin into rabbits causes either no change or

only a relatively slight increase in the oxidation of carbohydrates (Macleod, 1926). This conclusion has in general been confirmed by other workers using rabbits. The results differ from those on normal dogs, on eviscerated cats (Burn and Dale, 1924-25), and on diabetic animals, in all of which there is evidence that insulin causes considerable increase in carbohydrate combustion. They also differ from those obtained on small animals, such as rats and mice, in which insulin depresses the O_2 consumption, although it temporarily raises the r.q. (Dudley, Laidlaw, Trevan, and Boock, 1923).

It is necessary to consider the causes for the discrepancy between these results and those of the present investigation. With reference to the results on dogs it is very important to emphasize that the increase in O_2 consumption following insulin does not occur, or does so only to a slight degree, when glucose is administered along with the insulin, either by subcutaneous injection or by mouth. On account of this result Dickson, Eadie, Macleod, and Pember (1924) concluded that the striking increase in O_2 consumption which follows when insulin is given alone to these animals must be due to the increased muscular activity which culminates in convulsions. This conclusion was substantiated by observing that the increase in O_2 consumption which follows the injection of insulin alone did not occur during the 1st hour or so following the injection but only later when, in many of the animals, the incidence of the rise could be seen to be associated with the hyperexcitability and increased muscle tone which precede the onset of convulsions.

Although the increase in r.q. which follows injection of insulin into diabetic (depancreatized) dogs, especially when carbohydrate is also given the animal, is particularly striking, it may be pointed out that more work must be done on such animals before it can be stated just exactly how much extra carbohydrate insulin causes them to burn.

The most striking difference in results shows itself in the decided increase which insulin causes in the O_2 consumption of the isolated perfused muscles of the cat, as compared with the small and doubtful increase in the present observations on the rabbit. It is important, in considering the possible cause of these differences, to

see whether the total energy metabolism of the muscles is of similar magnitude in these two types of experiment.

Burn and Dale (1924-25) as a result of observations on decapitated cats from which all the abdominal viscera had been removed (with the exception in some of the experiments of the liver) found in one of their observations (*cf.* their Table VII) that the rate of oxygen consumption corresponded to the oxidation of 0.200 gm. of carbohydrate per kilo and hour before insulin was injected and to 0.250 gm. per kilo and hour after its injection. In another animal the rate of sugar consumption after insulin was 0.34 gm. per kilo and hour. This represents a much smaller consumption of the carbohydrate than that observed by us in rabbits during absorption of carbohydrate; namely, 0.76 to 0.98 gm. per kilo and hour. The difference may be due to greater activity of the muscles in our experiments but in any case it is important to note that it does exist.

Another difference between the two groups of experiments is that much larger doses of insulin were used in the cat experiments, the fall in blood sugar being usually prevented by the continuous intravenous injection of glucose. By this technique the great advantage is gained that the actual amount of glucose which disappears in a given interval can be estimated, by adding the amount of glucose injected to the amount which is meanwhile found to disappear from the blood and other circulating fluids. To obtain this latter value it was considered by these authors that the total volume of circulating fluids in a cat of 3 kilos amounts to 500 cc. and that the percentage of sugar in all the body fluids is the same. Comparison of the total disappearance of glucose with the oxygen used following the injection of insulin showed that the latter was far short of the amount necessary to account for the disappearing glucose.

More recently, using the same type of preparation and calculating the actual disappearance of glucose by the method outlined above, with allowance for glucose derived from the liver when this organ was not completely eliminated, Best, Dale, Hoet, and Marks (1926) showed that that portion of the disappearing glucose which could not be accounted for by increased oxidation was deposited in the muscles as glycogen. After concluding that "with a preparation consisting essentially of naturally perfused quiescent

skeletal muscle, all the glucose disappearing under insulin is either oxidized or stored in the muscles as glycogen," they hasten to point out that this does not account for all the effects of insulin on normal animals. The tendency for the glycogen of the body to be decreased rather than increased and the fall in oxygen consumption, which are commonly observed to follow insulin injection in the smaller laboratory animals, are cited as evidence to show that other factors must be concerned.

Before proceeding to discuss the possible nature of these factors it is important to point out that while there can be no doubt about insulin causing glycogen deposition in the muscles of the eviscerated preparation, as used by the above investigators, this does not indicate the degree to which increased glycogen formation is a factor in accounting for the disappearance of carbohydrate following the injection of insulin in normal animals. In collaboration with Orr and Barbour (Barbour, Chaikoff, Macleod, and Orr, 1927) we have recently shown that insulin in subconvulsive doses always caused the glycogen of the liver and muscles to *decrease* in fasting white rats and that very large, or repeated doses given to similar animals while they are absorbing large amounts of carbohydrate cause glycogen formation in the liver to be retarded while that in the muscles is increased. Even in the latter case, however, it is difficult to account for much of the disappearing carbohydrate as being deposited as glycogen, since the deficit deposited in the liver very nearly balances the excess deposited in the muscles.

The changes occurring in the muscles of an eviscerated animal may not correspond to those in an intact one. Quite apart from the relatively much smaller consumption of energy in the spinal preparation, which has already been referred to, there is evidence of some difference in the type of metabolism in the two cases. Thus, Best and his coworkers found that no change occurred in the glycogen content of corresponding muscles of the two legs after those of one side had for some time been perfused with blood containing high percentages of glucose. This result has been confirmed by Choi working in this laboratory. But this does not indicate that glycogen was not being formed by these muscles, since they must meanwhile have been using carbohydrate, as indicated by the O_2 absorption and an R.Q. of unity.

Where did this carbohydrate come from? It must have been either glucose taken directly from the blood, or muscle glycogen. Presumably it was the latter, and in such a case it would be necessary to assume in their experiments that new glycogen was being formed and added to the supply of each muscle just exactly at the same rate as this was disappearing.

In any case it is certain that increased oxidation and increased glycogen formation fall far short of accounting for all the carbohydrate which is often observed to disappear in intact animals as a result of insulin (see, for example, the results of Heymans and Matton (1924), Tsubura (1924), and Eadie and Macleod (1923). The question is what is the nature of the process responsible for the disappearance of the balance. One of us has suggested that much of the disappearing carbohydrate becomes converted into some intermediary metabolite which has lost the chemical properties both of a reducing sugar and of glycogen, so that it fails to be detected by the usual methods of analysis of the tissues. It is theoretically possible that this material might contain relatively less oxygen than carbohydrate, its formation thus accounting for the slight rise in R.Q. which follows administration of insulin. Diligent search of the muscles and liver for evidence of the appearance of such a substance, following injection of insulin and sugar, has, however, yielded only negative results (Macleod and Simpson, 1926) and it has not been possible to detect any increase in fat (Raper and Smith, 1925).

In the entire absence of any direct evidence that such a substance is formed it becomes necessary to seek for some other possible explanation for the discrepancy between the two sides of the carbohydrate balance sheet when insulin is given. A possible one originally suggested by Laufberger (1924) and endorsed by Best *et al.* is that insulin may inhibit the process of gluconeogenesis, by which glucose is constantly being formed in the liver out of protein and, probably, fat. This is the form now taken by the original hypothesis of Dale that insulin acts by causing a qualitative change in metabolism so that a higher proportion of carbohydrate is metabolized. According to present day views, liberation of energy in the muscles is exclusively dependent on oxidation of their carbohydrate (glycogen), which is supplied to them as blood glucose. This arises in the liver by hydrolysis of glycogen formed out of

glucose, which is derived from the digestive tract, or is manufactured out of fat and protein by the liver cell. It is evident that the R.Q. of the oxidative process in the muscles will be 1.00, whereas that of the gluconeogenic process in the liver will be somewhere about 0.3, the quotient of the animal as a whole being therefore the algebraic sum of these two quotients. If insulin should depress the gluconeogenic process the R.Q. must become increased, which as a matter of fact is the case, to a certain degree at least, in fasting or moderately fed animals. The quotient does not increase sufficiently however to make it permissible, as Laufberger has done, to consider that inhibition of gluconeogenesis is the sole effect of insulin. Accepting this process as one factor in the action of insulin on normal animals, Best, Dale, *et al.* explain the decrease in respiratory metabolism and the subsequent fall in temperature which insulin causes in small animals, such as mice, to be due to the rapid exhaustion of the stores of carbohydrate originally available in the body, no new supply being forthcoming because of inhibition of gluconeogenesis. They find support for this view in the fact that diabetes is largely due to an exaggeration of the gluconeogenic process on which insulin has a moderating influence. Its action on the normal animals is therefore believed to be of essentially the same nature as that in the diabetic animal.

While unquestionably inhibition of gluconeogenesis is an important factor in the action of insulin in the diabetic animal we doubt whether such inhibition can occur in normal animals injected with this hormone to a degree sufficient to account for that portion of the disappearing carbohydrate which cannot be attributed to increased combustion and increased glycogen formation even when these processes are stimulated. For example, it is impossible that inhibition of gluconeogenesis could have been a factor in lowering the blood sugar in those experiments of the present investigation in which insulin was given to rabbits in whose tissues maximal quantities of glycogen had (presumably) been deposited, by rich carbohydrate feeding, and in which, as judged from the R.Q., no gluconeogenesis could have been occurring prior to the injection of insulin. In this group as shown in Table I there was practically no change either in R.Q. or in the extent of carbohydrate consumption over a period of 6 hours following the injection of a large dose of insulin

(10 units). The only possible explanation for the carbohydrate which disappeared is therefore, either that it was deposited as glycogen, or that it was converted into something else. We cannot of course be certain that the glycogen stores were really full to their capacity before insulin was given, but it seems unlikely that sufficient extra glycogen could have been deposited in them to account for the disappearing sugar. In any case it is clear that under the conditions of this experiment no process of gluconeogenesis was occurring which could be inhibited, and there is no evidence that more carbohydrate was oxidized.

When insulin was given to fasting rabbits there was, in every one of the five experiments, evidence of a distinct increase in the amount of carbohydrate oxidized, but it was marked only in one case (November 22, Rabbit XI) in which 0.84 gm. of extra carbohydrate was metabolized in 6 hours, or 0.14 gm. per kilo and hour. This compares well with the previous findings of Dickson *et al.* who, in two animals (Table VI) in which the respiratory quotients before insulin were about 0.8, found that insulin caused 0.102 and 0.089 gm. of glucose per kilo and hour, respectively, to be oxidized. If we allow that an average of 50 mg. of glucose disappeared from each 100 cc. of blood as a result of insulin during each hour of its action the amount disappearing from the blood alone might be accounted for by the increased combustion in only one of the present group of experiments. But this does not take into consideration the glycogen also disappearing from the muscles and liver. That insulin increases the respiratory exchange more decidedly in meat-fed, as compared with carbohydrate-fed rats, has also been noted by Abderhalden and Wertheimer (1924).

There is nothing in our results on fasting animals which would contradict the view that insulin causes inhibition of gluconeogenesis and, as pointed out by Best *et al.*, if the dose be sufficient so that the inhibition is marked then the glycogen stores of the muscles will soon become exhausted and the body temperature will fall from want of fuel, which, as is well known, is exactly what happens in the case of small animals.

In neither the fed nor the fasted animal was there evidence of any significant increase in energy metabolism, for although this occurred in some cases a corresponding decrease occurred in others. Now such increase in energy output would be inevitable in the fed

animals if we assume, on the one hand, that combustion of carbohydrate is the only energy-producing process occurring in the muscles and, on the other, that insulin stimulates it. This narrows us down to increased glycogen formation as the process accountable for the disappearing carbohydrate, but we know that such does not occur in fasting normal animals and it seems reasonable to argue that it is unlikely to occur in the animal as a whole in those that have been liberally fed.

It is impossible here to refer in detail to the numerous observations by other workers on the influence of insulin on the respiratory exchange but some reference must be made to the work of Hawley and Murlin (1925-26). They used rabbits 19 hours after withdrawal of food, when they were in a basal condition as evidenced by the R.Q. After recording the metabolism for one or two basal periods subconvulsive doses of insulin were injected and the respiratory observations were continued for 45 minute periods at intervals up to 5 hours.

The energy output varied in the basal condition between 3.24 and 3.92 calories per kilo and hour (their Table II) whereas our corresponding values for fasting rabbits varied between 2.52 and 2.92 and for fed ones, between 2.83 and 3.68 calories. These differences are no doubt to be accounted for by there having been abundance of glycogen available in the more recently fed rabbits of the Rochester workers. Taking the average of their results they concluded that insulin causes O_2 intake to decrease in the second period, while in both the periods CO_2 excretion increases so that the R.Q. becomes raised in the second period.

The average of each hour in our experiments (*cf.* curves *B* and *C* of Fig. 1) shows, in fasted animals, a decided increase in both O_2 and R.Q. at the end of the 1st hour after insulin. This increase persists to the end of the 2nd hour, when the R.Q. falls back to the normal level although the O_2 continues to rise to the end of the 3rd hour. In fed animals (*cf.* curves *A*) the average of the R.Q.'s remains unchanged at unity and there is also no change in O_2 at the end of the 1st hour although it is somewhat increased during the 2nd, 3rd, and 4th hours.

Hawley and Murlin interpret the immediate increase in O_2 consumption as due to an early calorogenic action of insulin, but it may be pointed out that if this were so the same increase in

O₂ consumption ought to occur in fed animals, which our results for the 1st hour following insulin fail to show. Moreover, when insulin is injected along with epinephrine it is said to prevent the increased O₂ consumption which injection of the latter hormone alone brings about. If the action of epinephrine on O₂ absorption is calorogenic, that of insulin cannot also be so.

These authors also conclude that there is a diminished O₂ consumption during the second period of insulin action which they explain is due to additional oxygen being "made available for combustion (heat production) through a change to an oxygen-rich foodstuff," and they consider that this confirms the view that insulin has caused a shift in metabolism. While, as we have already stated, we agree that such a shift may take place we cannot confirm this evidence of its occurrence. In both our groups of animals the O₂ consumption following insulin remained increased at least until the end of the 2nd hour and in any case supposing even that O₂ consumption did decrease with a rise in R.Q. it would only mean that more carbohydrate was being consumed in the muscle while less was being manufactured out of protein and fat in the liver.

Finally these authors consider that their results indicate that the increased combustion of carbohydrate is much more than sufficient to account for the sugar which insulin causes to disappear from the blood and tissue fluids, but they show this increase to occur not during the 1st but during the 2nd hour after insulin injection. They attempt to explain this anomalous result by invoking the idea of a calorogenic action of insulin and they think that direct calorimetry ought to confirm this.

CONCLUSIONS.

1. When administered to rabbits in which, by continued feeding with carbohydrates, the R.Q. stood at unity, insulin caused no significant increase in O₂ consumption and no change in R.Q. Under these conditions of feeding there is therefore no evidence that insulin causes increased combustion of carbohydrates. Since gluconeogenesis was not occurring in these animals (as judged by R.Q.) the sugar which insulin caused to disappear must either have been deposited as glycogen, or converted into some unknown in-

termediary substance, or it may have been dealt with by both processes. It is unlikely that glycogen could have been formed to any great extent since the glycogen depots must already have been filled on account of the feeding.

2. When administered to the same rabbits while in a fasting condition insulin caused a rise in both O_2 consumption and R.Q. The rise amounted, on an average, to 0.35 gm. of carbohydrate, which according to the usual methods of calculation indicates that somewhat below 10 per cent more of the total calorie expenditure came from carbohydrate after insulin.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.

V. THE COMPOSITION AND RESPIRATORY EXCHANGES OF NORMAL HUMAN BLOOD DURING WORK.

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This paper presents the results of further studies of the blood of a normal man, A. V. B. In the second number of this series (1) his resting blood was described. The present investigation concerns the blood as it exists in the steady state during exercise on a stationary bicycle, the oxygen consumption being approximately 1750 cc per minute; *i.e.*, 7 times the value for rest. In all cases blood was drawn for analysis after a period of not less than 15 minutes of uniform work and while work was being continued.

As before, the first step in the investigation consisted of analyses to provide data for the construction of the oxygen and carbon dioxide dissociation curves. For the latter, samples of both arterial and venous blood were employed. Significant differences in any experiment utilized in constructing the nomogram between the two kinds of blood were not observed. These curves are presented in Figs. 1 and 2. During the past 5 years many analyses have been made of the blood of A. V. B., the shape of the curves is well known, and accordingly a relatively small number of analyses suffice in order to determine the position of the curves.

The physiological adjustments which accompany work include a number of concentration changes in the blood. There are recorded in Table I, for three normal subjects and for various metabolic levels, some of the more important of these changes; *viz.*, oxygen capacity of whole blood, serum protein, serum base, cell base, serum water, and cell water.

A great number of observations on A. V. B., only a few of which are included in Table I, indicate that in heavy work there is an increase in the oxygen capacity of his blood of approximately 10 per cent. During work on February 17, 1926, the capacity was 22.1 volumes per cent. It was on this date that complete data for the

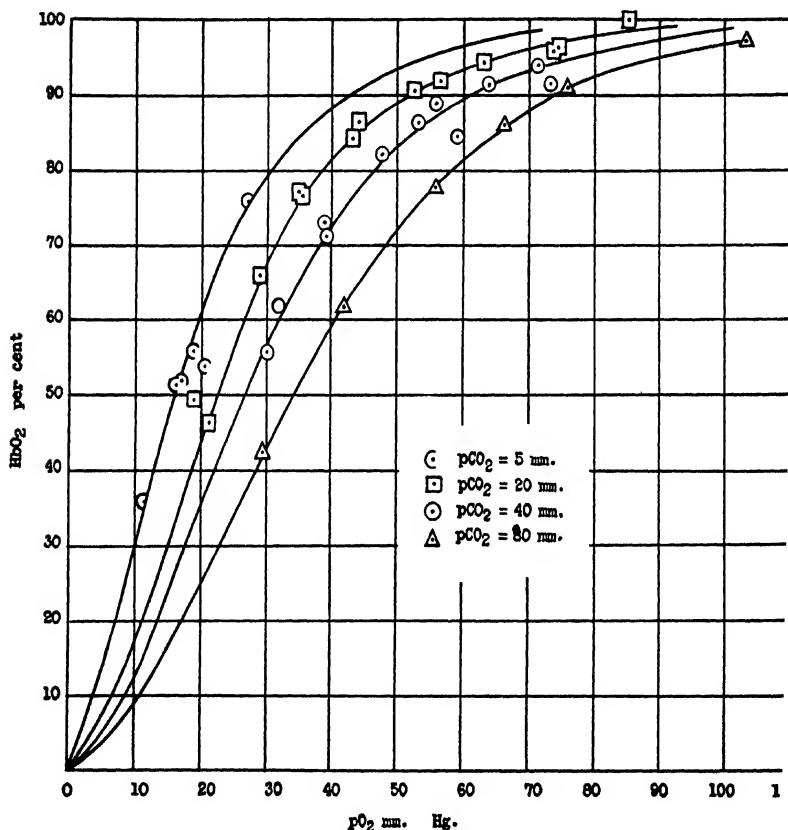


FIG. 1. Oxygen dissociation curves of blood of A. V. B. at work.

carbon dioxide dissociation curves were obtained and at this period his resting oxygen capacity was approximately 20 volumes per cent. Consequently, in construction of the nomogram, a value of 22 volumes per cent has been assigned as the capacity of his blood during work. It is not certain that this is the commonest response

to a sevenfold increase in metabolism. For a given increase in the metabolic rate, there appears to be a greater increase in the oxygen capacity of the blood of A. V. B. than in that of D. B. D. or of L. M. H.

The total nitrogen content of the serum was determined by Kjeldahl's method. Non-protein nitrogen was determined by the

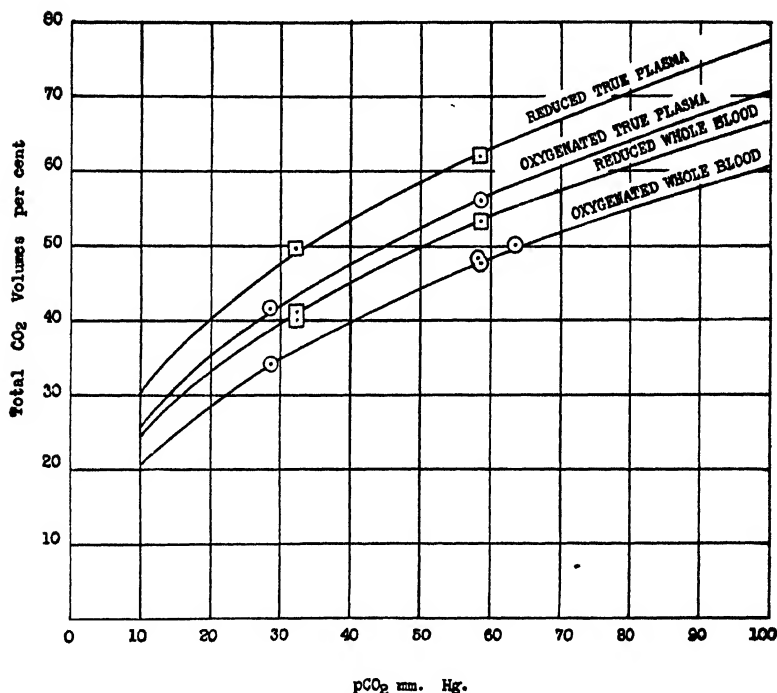


FIG. 2. Carbon dioxide dissociation curves of blood of A.V.B. at work.

method of Folin and Wu (2). The difference was assumed to be protein nitrogen and the factor 6.25 was employed to calculate total protein. The data given in Table I indicate that for A. V. B. at rest the concentration of serum protein is approximately 72.5 gm. per liter of serum. Working at an oxygen consumption of 1700 to 2000 cc. per minute, the average concentration is about 80 gm. per liter, a 10 per cent increase. This quantity is subject to con-

TABLE I.
Concentration Changes in Serum, Cells, and Whole Blood in the Adjustment from Rest to Work.

Date.	Subject.	Conditions of experiment	Oxygen used per minute	Oxygen capacity	Serum protein	Serum base	Cell base	Serum water	Cell water
			cc	vol per cent	gm per l serum	mM per l serum	mM per l cells	gm per l serum	gm per l cells
1938									
Nov.									
8	D.B.D.	Working 22 min	1340	18 05	73 6				
8	A.V.B.	" 23 "	1640	21 96	69 6				
9	L.M.H.	" 21 "	1435	20 42	68 7				
12	D.B.D.	" 22 "	1139	18 66	73 5				
17	"	" 10 "	2285	18 81	77 7	161			
17	"	" 33 "	2268	18 77	81 9	158			
23	L.M.H.	Resting	340	20 21	66 5	157			
23	"	Working 60 min.	1060	20 97	71 0				
30	"	Resting.	300	20 24	66 8				
30	"	Working 27 min.	1454	21 15	67 5				
30	D.B.D.	Resting.	250	17 18	69 8				
30	"	Working 26 min.	1678	18 22	75 2				
Dec.									
7	"	Resting.	250	19 32	73 8				
7	"	Working 30 min.	1466	19 43	77 6				
10	"	" 19 "	2360	20 55	82 2				
27	"	" 22 "	2268	19 43	81 0				

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siderable variation. Changes in the quantities of other osmotically inert constituents were not studied.

Serum water and cell water were determined gravimetrically. It was found that the increase in concentration of serum proteins is associated with a decrease in serum water from 943 to about 938 gm. per liter of serum. No evidence was found of any change in the concentration of solids within the cell.

The concentration of base in the serum of A. V. B. was determined by the method of Fiske (3) and found to be 154 mm per liter of serum at rest. In work the concentration in his serum was increased to about 160 mm per liter. In the serum of D. B. D. and

TABLE II
Chloride Concentration in Serum and Cells of A. V. B. at Rest and at Work

Date	Oxygen used per minute	Oxygen capacity	Total CO ₂ of whole blood at $p\text{CO}_2 = 40$ mm	$p\text{CO}_2$	$p\text{O}_2$	pH_s	Serum chloride	Cell chloride	Cell volume
	cc	vol per cent	vol per cent	mm	mm		mm per l serum	mm per l cells	per cent
1927									
Jan.									
19	250	21 28	46 5	49 5	Air.	7 34	101 6	51 3	44.9
19	2467	23 16	40 9	27 5	"	7 42	106 1		46 7
				98 3	"	7 24	102 3	51 8	46 3
21	2150	22 58	40 9	23 7	"	7 48	104 4	47 7	46.3
				82 7	"	7 15	100 3	52 2	46 8
				22 5	10 9	7 53	102 2	50 6	47 7
				90 0	11 1	7 16	97 3	57 1	47.6

L. M. H. there was a similar change. The observations on cell base are too few and too inconsistent to be relied upon. We have employed, therefore, the equation of Van Slyke, Wu, and McLean (4) for calculation of cell base.

The method of Van Slyke (5) was used for determination of chlorides in serum and in cells. The values for chloride concentration in serum and in cells of equilibrated blood are shown in Table II. Since these concentrations are expressed as mm per liter of serum and of cells, respectively, it is necessary, in order to calculate r , to establish the relation between pH_s and $V_s:V_c$ and between pH_s and $(\text{H}_2\text{O})_s$. With the limited amount of blood at

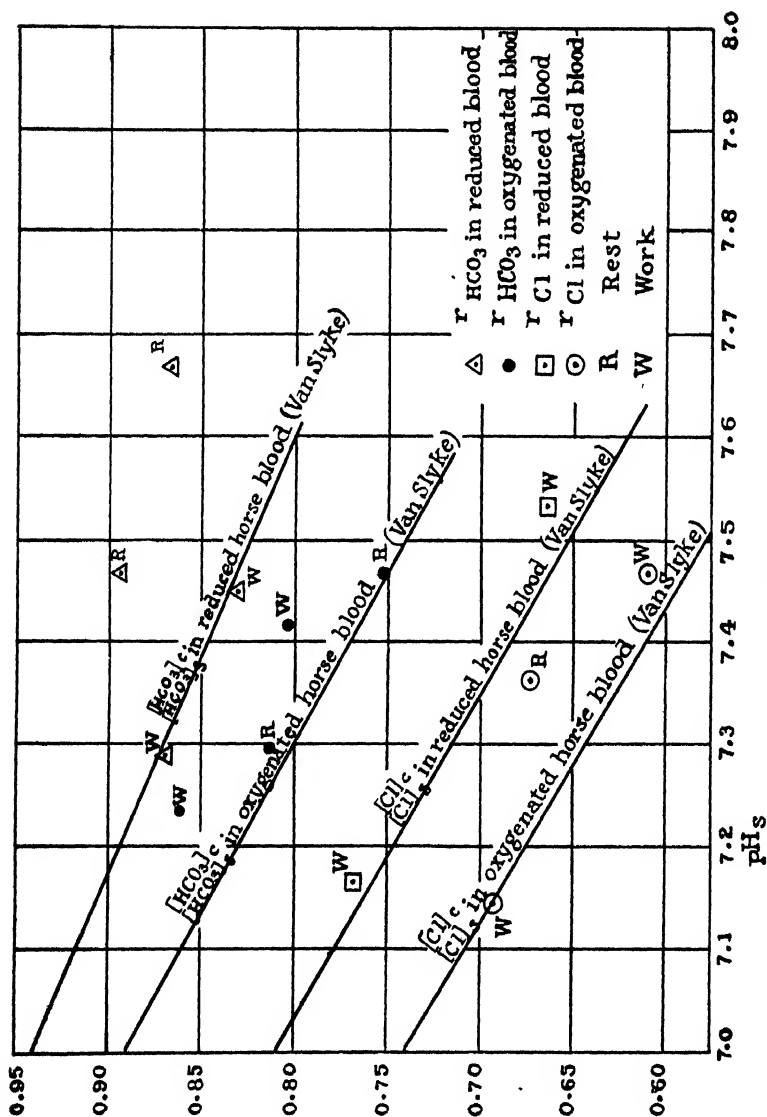


Fig. 3. r as a function of pH.

our disposal, it has been impossible to determine these relationships even approximately. We have relied, therefore, upon the equations developed by Van Slyke, Wu, and McLean (4) for calculation of the change, both in oxygenated and in reduced blood, of $V_s:V_b$ and $(H_2O)_s$ with pH_s . From these calculations and from the data of Table II, r_{Cl} has been calculated. The values found are shown in Fig. 3. It may be seen that these values agree closely with the experimental values for horse blood found by Van Slyke, Hastings, Murray, and Sendroy (6). In the same figure the values for r_{HCO_s} are shown. These ratios are calculated from smoothed values read from the carbon dioxide dissociation curves of whole blood and plasma. No great accuracy is attributed to

TABLE III.
Lactate and Bicarbonate Changes in Whole Blood from Rest to Work.

Subject	Conditions of experiment	Oxygen used per minute	Lactate	Δ lactate from rest to work	Δ bicarbonate from rest to work.
		cc.	mm per l blood	mm per l blood	mm per l blood
D.B.D.	Working 15 min.	2474	8 9	+7 2	-4 1
"	Resting.	250	1.7		
"	Working 20 min.	820	1 3	-0 3	+0 4
"	" 15 "	2000	6 1	+4 4	-1.3
"	" 60 "	2000	4 8	+3 1	-0 3
A.V.B.	Resting.	250	2 1		
"	Working 42 min.	1925	8 9	+6 8	-3 1
"	" 16 "	2315	8 9	+6 8	-5 3

these ratios since the concentration of bicarbonate in the cells is calculated by difference.

A few determinations have been made of the concentration of lactic acid in whole blood at various metabolic levels. The method of Clausen (7) was employed. The results, expressed in mm of lactate per liter of blood, are shown in Table III. A comparison is made in this table of the increase in lactate and decrease in bicarbonate which occur with increased metabolism. It is clear that in general the increase in lactate is much more than equivalent to the decrease in bicarbonate.

In Figs. 4 and 5 the level of the carbon dioxide dissociation curve of oxygenated whole blood at 40 mm. of carbon dioxide pressure

is plotted against the metabolic rate expressed as oxygen consumption per minute. The data are sufficiently numerous to prove that the changes in the blood are not uniformly of the same magnitude even in the same individual. It appears that with a sevenfold

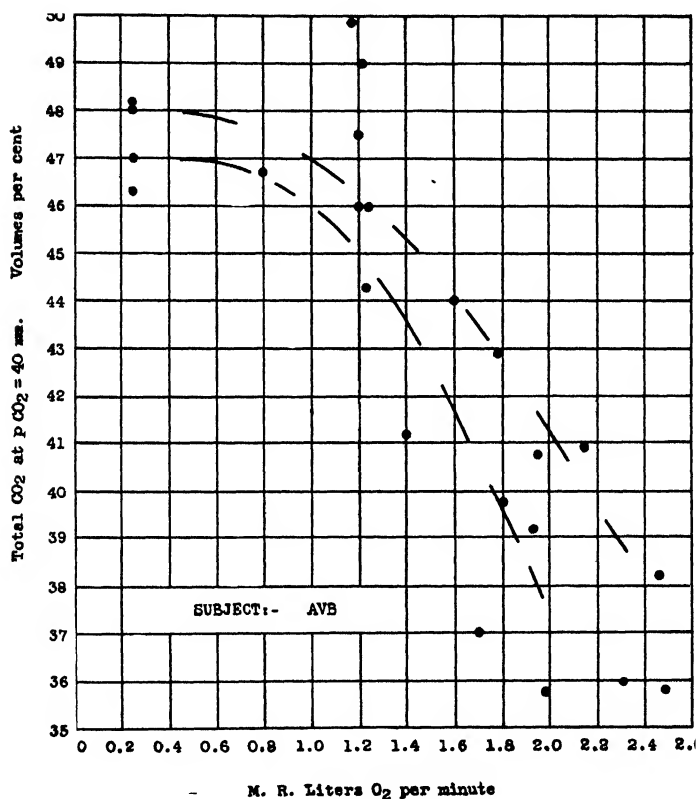


FIG. 4. Level of carbon dioxide dissociation curves of the oxygenated whole blood as a function of metabolic rate in A.V.B.

increase in metabolic rate the blood of D. B. D. and L. M. H. undergoes no significant decrease in bicarbonate. So far as the level of the carbon dioxide dissociation curves is concerned, a ninefold increase in the metabolism of D. B. D. is approximately equivalent to a sevenfold increase in the metabolism of A. V. B.

With the help of the dissociation curves, Figs. 1 and 2, and these data the nomogram, Fig. 6, has been constructed in accordance

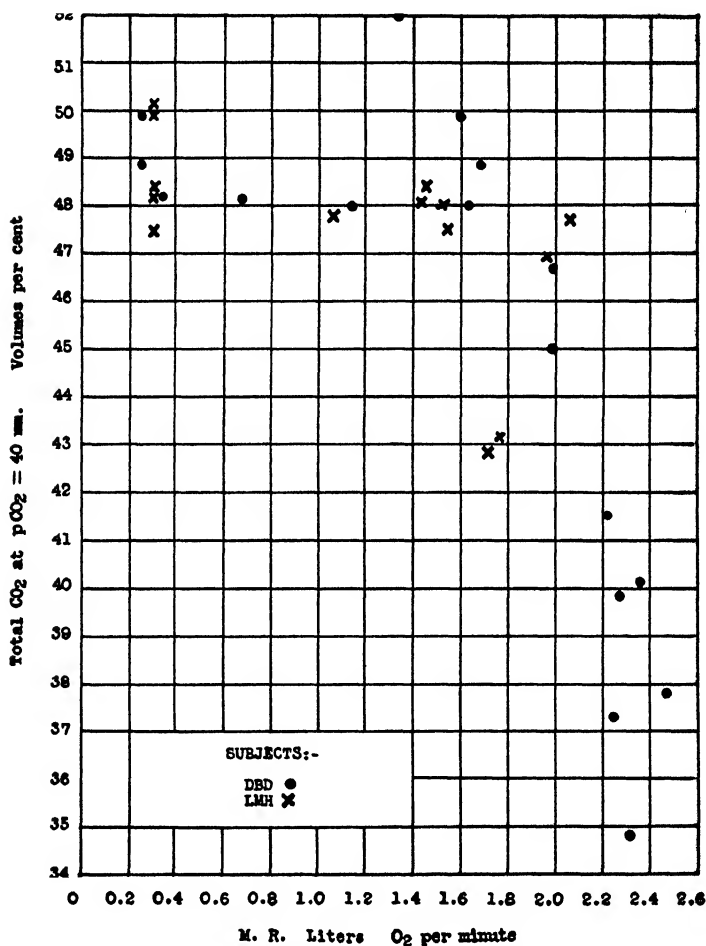


FIG. 5. Level of carbon dioxide dissociation curves of the oxygenated whole blood as a function of metabolic rate in D.B.D. and L.M.H.

with the methods described in earlier papers. The position of the arterial and venous lines which are drawn on the nomogram was determined by studies of alveolar air and of air believed to be in

[illegible]

FIG. 6. Blood of A.V.B. at work.

printed as Fig. 7. In this nomogram certain slight changes have been introduced in order that the comparison may represent the best values that we possess of resting blood of A. V. B. at the time of the work experiments.

Figs. 6 and 7 describe the more important properties of the blood at rest and at work under the conditions specified, and give directly the changes in the blood accompanying the change in activity. In order to complete the description it is sufficient to tabu-

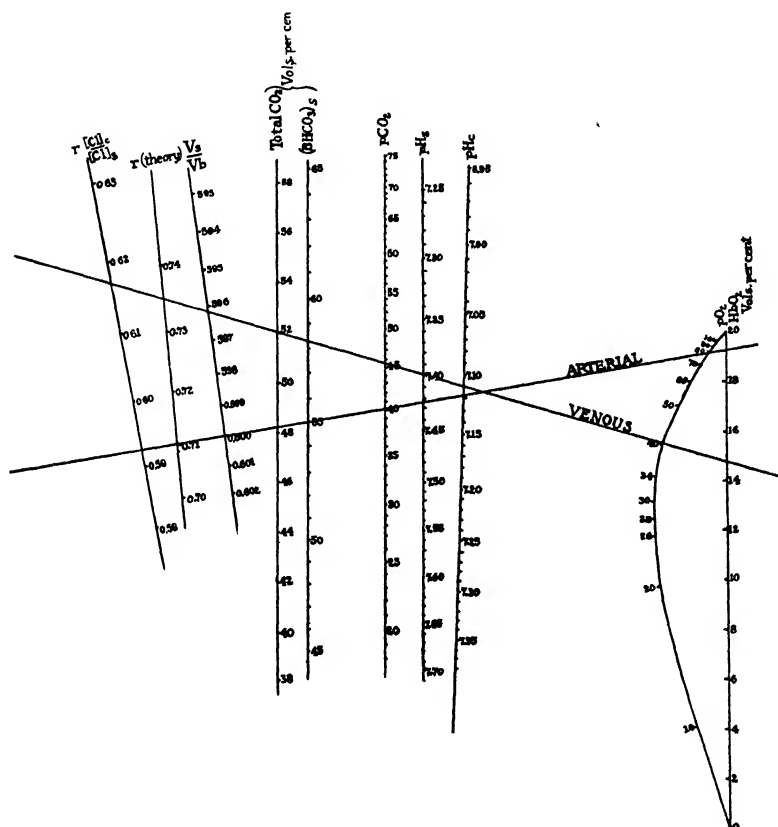


FIG 7. Blood of A.V.B at rest.

late the values of the numerous variables, of their differences, etc., for the two cases.

Table IV comprises the values of the variables for the working state in arterial blood and in mixed venous blood, together with the differences measuring the physiological respiratory changes. Table V gives the corresponding values for resting blood.

Blood of A.V.B. at Work.

Concentration of hemoglobin = 9.82 mm per liter of blood.
" " serum proteins = 44.8 gm " "
Respiratory quotient = 1.00

	Arterial			Venous			A		
	Serum	Cells.	Whole blood	Serum	Cells.	Whole blood	Serum	Cells	Whole blood.
H ₂ O cc. per l. blood.	525.0	310.5	835.5	517.6	317.9	835.5	-7.4	+7.4	0.0
B mm " " " "	89.60	58.37	147.97	89.60	58.37	147.97	0.0	0.0	0.0
X " " " " "	13.31	8.83	22.14	13.23	9.09	22.32	-0.08	+0.26	+0.18
Cl " " " " "	57.48	21.77	79.25	55.44	23.81	79.25	-2.04	+2.04	0.0
BP " " " " "	7.79	22.39	30.18	7.57	18.41	25.98	-0.22	-3.98	-4.20
BHCO ₃ " " " " "	11.02	5.38	16.40	13.36	7.06	20.42	+2.34	+1.68	+4.02
" vol. per cent ..	24.69	12.05	36.74	29.94	15.80	45.74	+5.25	+3.75	+9.00
H ₂ CO ₃ mm per l. blood..	0.65	0.38	1.03	0.92	0.56	1.48	+0.27	+0.18	+0.45
" vol. per cent.	1.45	0.86	2.31	2.06	1.26	3.32	+0.61	+0.40	+1.01
Total CO ₂ mm per l. blood.	11.67	5.76	17.43	14.28	7.62	21.90	+2.61	+1.86	+4.47
" " vol. per cent ...	26.14	12.91	39.05	32.00	17.06	49.06	+5.86	+4.15	+10.01
Free O ₂ mm per l. blood			0.09			0.04			-0.05
" " vol. per cent			0.19			0.08			-0.11
Combined O ₂ mm per l. blood....			9.33			4.91			-4.42
" " vol. per cent ..			20.90			11.00			-9.90
Total O ₂ mm per l. blood..			9.42			4.95			-4.47
" " vol. per cent			21.09			11.08			-10.01
pO ₂ mm. Hg.....			38.0			54.8			+16.8
pO ₂ " "			75.0			31.0			-44.0
Volume, cc. per l. blood	559.5	440.5	1000.0	552.6	447.4	1000.0	-6.9	+6.9	0.0
pH.....	7.351	7.062		7.278	7.027		-0.073	-0.035	
p _H theory			0.740			0.793			+0.053
pHCO ₃			0.825			0.859			+0.034
pCl.....			0.641			0.699			+0.058

TABLE V.
Blood of A. V. B. at Rest.
 Concentration of hemoglobin = 8.93 mm per liter of blood.
 " serum proteins = 43.5 gm. " " "
 Respiratory quotient = 0.82

	Arterial			Venous			Δ		
	Serum	Cells	Whole blood	Serum	Cells	Whole blood	Serum	Cells	Whole blood
H ₂ O cc. per l. blood	566	282	848	562	286	848	-4	+4	0 0
B " " "	92.40	53.50	145.90	92.40	53.50	145.90	0 0	0 0	0.0
X " " "	10.20	7.09	17.29	10.38	6.91	17.29	+0.18	-0.18	0.0
Cl " " "	59.59	18.11	77.70	58.72	18.98	77.70	-0.87	+0.87	0.0
BP " " "	7.88	22.60	30.48	7.80	21.15	28.95	-0.08	-1.45	-1.53
BHCO ₃ " " "	14.73	5.70	20.43	15.50	6.46	21.96	+0.77	+0.76	+1.53
" vol. per cent	33.00	12.77	45.77	34.71	14.49	49.20	+1.71	+1.72	+3.43
H ₂ CO ₃ mm per l. blood	0.73	0.37	1.10	0.83	0.42	1.25	+0.10	+0.05	+0.15
" vol. per cent	1.64	0.82	2.46	1.86	0.94	2.80	+0.22	+0.12	+0.34
Total CO ₂ mm per l. blood	15.46	6.07	21.53	16.33	6.88	23.21	+0.87	+0.81	+1.68
" " vol. per cent	34.64	13.59	48.23	36.57	15.43	52.00	+1.93	+1.84	+3.77
Free O ₂ mm per l. blood			0.09			0.04			-0.04
" " vol. per cent			0.2			0.1			-0.1
Combined O ₂ mm per l. blood		8.57	8.57		6.56	6.56		-2.01	-2.01
" " vol. per cent		19.2	19.2		14.7	14.7		-4.5	-4.5
Total O ₂ mm per l. blood			8.66			6.60			-2.05
" " vol. per cent			19.4			14.8			-4.6
pCO ₂ mm. Hg. . . .			40.0			45.4			+5.4
PO ₂ " " . . .			78.0			40.0			-38.0
Volume, cc. per l. blood	600	400		596.2	403.8	40.0	-3.8	+3.8	0.0
pH.	7.425	7.118		7.399	7.110		-0.026	-0.008	+0.025
rtheory			0.710			0.735			+0.025
rCl			0.610			0.635			+0.025
rHCO ₃			0.777			0.820			+0.043

TABLE VI.
Serum of A.V.B. at Work.

		Arterial.	Venous.	Δ	$\frac{100\Delta}{A}$
H ₂ O	cc. per l. serum . .	938 4	936.7	-1.7	-0.2
B	mM " " "	160.1	162.1	+2 0	+1.2
X	" " " "	23 78	23 92	+0.14	+0.6
Cl	" " " "	102.7	100 3	-2.4	-2.3
BP	" " " "	13 92	13 70	-0.22	-1.6
BHCO ₃	" " " "	19 70	24.18	+4.48	+22.7
H ₂ CO ₃	" " " "	1.16	1 66	+0.50	+43 0
Total CO ₂	" " " "	20 86	25 84	+4 98	+23.9

TABLE VII.
Cells of A.V.B. at Work.

		Arterial	Venous	Δ	$\frac{100\Delta}{A}$
H ₂ O	cc per l. cells....	704 9	710 5	+5.6	+0.8
B	mM " " "	132 5	130 5	-2 0	-1.5
X	" " " "	20 04	20 32	+0 28	+1.4
Cl	" " " "	49 42	53 22	+3 80	+7.7
BP	" " " "	50 83	41 15	-9 68	-19 0
BHCO ₃	" " " "	12 22	15 78	+3 56	+29 1
H ₂ CO ₃	" " " "	0 86	1.25	+0 39	+45 5
Total CO ₂	" " " "	13 08	17 03	+3 95	+30 2
Combined O ₂	" " " "	21 18	10 97	-10.21	-48 4

TABLE VIII
Serum of A.V.B. at Rest.

		Arterial.	Venous	Δ	$\frac{100\Delta}{A}$
H ₂ O	cc. per l. serum	943 3	942.6	-0.7	-0.1
B	mM " " "	154.0	155.0	+1.0	+0 6
X	" " " "	17.00	17.41	+0.41	+2.4
Cl	" " " "	99.32	98.49	-0 83	-0 8
BP	" " " "	13.13	13.08	-0.05	-0.4
BHCO ₃	" " " "	24 55	26.00	+1 45	+5.9
H ₂ CO ₃	" " " "	1 22	1.39	+0.17	+13.9
Total CO ₂	" " " "	25 77	27.39	+1.62	+6.3

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TABLE IX.
Cells of A.V.B. at Rest.

		Arterial	Venous	Δ	$\frac{100\Delta}{A}$
H ₂ O	cc. per l. cells	705 0	708 3	+3.3	+0 5
B	mM " " "	133 75	132 5	-1.25	-0.9
X	" " " "	17 73	17 11	-0 62	-3.5
Cl	" " " "	45 27	47 00	+1 73	+3.8
BP	" " " "	56 50	52 38	-4 12	-7.3
BHCO ₃	" " " "	14 25	16 00	+1 75	+12.3
H ₂ CO ₃	" " " "	0 93	1 04	+0 11	+10 8
Total CO ₂	" " " "	15 18	17.04	+1 86	+12.2
Combined O ₂	" " " "	21 43	16.25	-5.18	-24.1

TABLE X
Arterial Serum of A.V.B. at Rest and at Work.

		Rest	Work.	Δ	$\frac{100\Delta}{\text{Rest}}$
H ₂ O	cc. per l. serum	943 3	938 4	-4 9	-0 5
B	mM " " "	154 0	160 1	+6 1	+4 0
X	" " " "	17 00	23 78	+6 78	+40 0
Cl	" " " "	99 32	102 70	+3 38	+3 4
BP	" " " "	13 13	13 92	+0 79	+6 0
BHCO ₃	" " " "	24 55	19 70	-4 85	-19 8
H ₂ CO ₃	" " " "	1 22	1 16	-0 06	-4 9
Total CO ₂	" " " "	25 77	20 86	-4 91	-19 1
pH	7.425	7 351	-0 074	

TABLE XI.
Arterial Cells of A.V.B. at Rest and at Work.

		Rest	Work	Δ	$\frac{100\Delta}{\text{Rest}}$
H ₂ O	cc. per l. cells.	705 0	704 9	-0 1	0 0
B	mM " " "	133.75	132.5	-1.25	-0 9
X	" " " "	17 73	20 04	+2.31	+13 0
Cl	" " " "	45 27	49 42	+4 15	+9 2
BP	" " " "	56 50	50 83	-5 67	-10.0
BHCO ₃	" " " "	14 25	12 22	-2 03	-14.2
H ₂ CO ₃	" " " "	0 93	0.86	-0.07	-7.5
Total CO ₂	" " " "	15.18	13.08	-2.10	-13.8
Combined O ₂	" " " "	21 43	21 18	-0 25	-1.2
pH	7.124	7.062	-0 062	

TABLE XII.

Respiratory Changes in Rest and Work.

	Serum				Cells			Whole blood.		
	I Rest.	II Work	II - I	II I	I Rest.	II Work	II - I	II - I	II Work	II - I
H ₂ O cc. per l. blood. . . .	-4.0	-7.4	3.4	1.85	+4.0	+7.4	3.4	1.85		
X " " " " " " " " " "	+0.18	-0.08	?	?	-0.18	+0.26	?	?	0.18	
Cl " " " " " " " " " "	-0.87	-2.04	1.17	2.34	+0.87	+2.04	1.17	2.34		
BP " " " " " " " " " "	-0.08	-0.22	0.14	2.75	-1.45	-3.98	2.53	2.74		
BHCO ₂ " " " " " " " " " "	+0.77	+2.34	1.57	3.05	+0.76	+1.68	0.92	2.18	-1.53	2.672.74
" vol. per cent	+1.71	+5.25	3.54	3.05	+1.72	+3.75	2.03	2.18	+1.53	2.492.61
H ₂ CO ₃ mm per l. blood.	+0.10	+0.27	0.17	2.70	+0.05	+0.18	0.13	3.60	+9.00	5.552.61
" vol. per cent. . . .	+0.22	+0.61	0.39	2.70	+0.12	+0.40	0.28	3.60	+0.15	0.302.97
Total CO ₂ mm per l. blood.	+0.87	+2.61	1.74	3.00	+0.81	+1.86	1.05	2.30	+1.68	0.672.97
" " vol. per cent . . .	+1.93	+5.86	3.93	3.00	+1.84	+4.15	2.31	2.30	+4.47	2.792.65
Free O ₂ mm per l. blood									+10.01	6.242.65
" " vol. per cent. . . .									-0.04	0.011.25
Combined O ₂ mm per l. blood. . .					-2.01	-4.42	2.41	2.20	-0.10	0.011.10
" " vol. per cent . . .					-4.50	-9.90	5.40	2.20	-4.42	2.412.20
Total O ₂ mm per l. blood. . . .									-2.05	5.402.20
" " vol. per cent. . . .									-4.47	2.422.18
pCO ₂ mm. Hg.									-4.60	5.412.18
pO ₂ " " " " " " " " " "									+5.4	11.4 3.11
Volume, cc. per l. blood	-3.8	-6.9	3.1	1.82	+3.8	+6.9	3.1	1.82	-38.0	6.0 1.16
pH	-0.026	-0.073	-0.047	2.80	-0.008	-0.035	0.027	4.4		
H ₂	+21.10 ⁻¹⁰	+78.10 ⁻¹⁰	+57.10 ⁻¹⁰	3.71	+14.10 ⁻¹⁰	+75.10 ⁻¹⁰	61.10 ⁻¹⁰	5.36		
r _{theory}									+0.025	0.0282.12
r _{HCO₃}									+0.034	+0.034
r _{Cl}									+0.025	0.0322.32

Tables VI and VII include the values of the important variables in the working state for cells and serum of arterial and venous blood. Tables VIII and IX give the corresponding values for the resting state.

In Tables X and XI the arterial serums and arterial cells of the working and resting state are compared.

In Table XII the respiratory *changes* (i.e. differences between arterial and venous blood) for serum, cells, and whole blood, at work and at rest, are compared.

A considerable number of observations on the blood of A. V. B. and of other normal individuals lead us to believe that the results of the present investigation are typical of those that may be generally observed during work. The changes in the blood are however not uniformly of the same magnitude even in the same individual. No doubt they depend upon physiological conditions which have not been taken into account in the present investigation. The facts in our possession which bear on this question are best illustrated by Figs. 4 and 5 discussed above. Since there are slight fluctuations in the level of the carbon dioxide curves from day to day these data illustrate the trend only of the effect of exercise upon the composition of blood.

SUMMARY.

This paper presents a description (1) of the principal physicochemical properties of the blood of a normal man in a steady state of work, (2) of the changes in the blood accompanying the change from rest to work.

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THE RELATION OF THE CONNECTIVE TISSUE CONTENT OF MEAT TO ITS PROTEIN VALUE IN NUTRITION.

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The relation of the connective tissue content of meat to its toughness has been established by the investigations of Lehmann (1), which showed that the toughness of raw meat depended largely upon its content of collagen and elastin fibers. In so far as the toughness was due to collagen fibers, it could be practically removed by cooking, resulting in the conversion of collagen to gelatin, although the rate of this conversion is evidently slow at ordinary cooking temperatures (2).

It appeared probable that the proportion of collagen and elastin in meats would also be related to their protein values in nutrition, as indicated, for example, by the biological values of their digestible nitrogen, since it is known that collagen, at least, is an incomplete protein. In so far as this relation exists, it would not be affected by cooking, since the conversion of collagen to gelatin would not presumably affect its nutritive value.

Before the investigation to be reported below was undertaken, we had at hand evidence of a circumstantial character that the more fibrous a cut of meat the lower the biological value of its nitrogen would be. Thus, it was found (3) that a cut of veal, evidently very fibrous when dried, ground, and sieved, gave a biological value of only 62, considerably lower than the values obtained with other meat samples; *i.e.*, 69 for a sample of beef (3) and 74 for a sample of pork (4). Similarly, in unpublished experiments, the biological value of the total nitrogen of a particularly tough and fibrous piece of beef, the lower round (heel) cut from a bull, was found to be only 56, not much higher than the value for white flour; *i.e.*, 52. The most plausible explanation of these variable values is the one suggested by the appearance of the

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different samples; namely, that an increased connective tissue content will decrease the value of the nitrogen in the nutrition of maintenance and growth.

The weakness of the argument lay in the absence of *quantitative* data relating to the connective tissue content of the various samples of meat used. With the working out of a method for the determination of the collagen and elastin content of meat (5) sufficiently accurate for the purpose, the opportunity of putting the question to a direct test was, for the first time, at hand. The plan of the experiment here reported was to determine the biological value of the nitrogen of (a) a cut of meat of low connective tissue content, (b) a sample of connective tissue itself, and (c) a definite mixture of the two, such as would be found in the less desirable cuts of meat.

The sample of meat chosen was a pork tenderloin which was ground in a sausage mill, dried at a low temperature, reground, and extracted with ether to remove the fat. The sample of connective tissue was prepared from pork adipose tissue (fat back). Most of the fat was rendered out of this material at a temperature of approximately 100°C. The pork cracklings thus obtained were ground and extracted with ether until practically fat-free.

The pork tenderloin was found to contain collagen equivalent to 2.7 per cent of its total nitrogen and elastin equivalent to 1.8 per cent. The corresponding values for the pork cracklings were 33.5 per cent (collagen) and 25.7 per cent (elastin).

In studying the value of these two animal products, alone and in a definite combination, as sources of protein for maintenance and growth, the biological values of their total digestible nitrogen were determined according to the routine adopted in this laboratory. The products were incorporated into a ration well balanced in all respects except for the absence of protein, to such an extent that the final ration contained approximately 8 per cent of crude protein ($N \times 6.25$). This required 8.68 per cent of the dried ether-extracted cracklings and 8.89 per cent of the dried ether-extracted pork tenderloin. Each ration also contained 4 per cent of the Osborne and Mendel (6) salt mixture, 4 per cent of Cellu Flour¹ as a source of roughage, 1 per cent of NaCl, 10 per cent of

¹ A product obtained from the Chicago Dietetic Supply House, containing 37.8 per cent crude fiber and 0.015 per cent nitrogen.

TABLE I
Nitrogen Metabolism Data and Biological Values.

Rat No.	Initial weight	Final weight	Food intake.	N intake	Fecal N	Urinary N	Biological value.	Rat No.	Initial weight	Final weight.	Food intake.	N intake	Fecal N.	Urinary N.	Biological value.
Period I. Egg ration, containing 0.70 per cent N.								Period I. Egg ration, containing 0.70 per cent N.							
	gm	gm	gm	gm	gm	gm	Per cent		gm	gm	gm	gm	gm	gm	per cent
451	50	57	7.2		9	10		461	52	59	7.5		9	12	
452	53	58	6.5		8	10		462	52	59	7.0		8	11	
453	55	61	7.8		9	13		463	50	57	7.1		9	10	
454	53	59	6.9		10	13		464	54	58	6.2		8	11	
455	61	68	7.4		8	13		465	56	62	6.5		8	10	
Period II. Pork tenderloin ration, containing 1.35 per cent N.								Period II. Pork crackling ration, containing 1.26 per cent N.							
451	67	79	8.0	108	10	36	80	461	54	48	3.7	47	10	51	4
452	70	82	8.0	108	9	34	81	462	53	46	3.4	43	9	53	0
453	69	81	8.0	108	9	38	80	463	53	45	2.8	35	10	47	0
454	68	81	8.0	108	9	39	78	464	53	47	3.1	39	9	49	0
455	80	92	8.0	108	9	39	78	465	59	52	2.6	33	9	40	0
Period III. Pork tenderloin-crackling ration, containing 1.34 per cent N.								Period III. Pork tenderloin-crackling ration, containing 1.34 per cent N.							
451	81	93	8.0	107	11	43	75	461	59	72	7.9	106	10	46	70
452	85	97	8.0	107	11	48	69	462	58	73	8.0	107	10	41	76
453	84	94	8.0	107	12	54	68	463	56	69	7.9	106	11	40	76
454	85	97	8.0	107	12	52	68	464	60	74	8.0	107	10	40	76
455	99	110	8.0	107	13	50	68	465	63	75	6.7	90	11	35	74
Period IV. Pork crackling ration, containing 1.26 per cent N.								Period IV. Pork tenderloin ration, containing 1.35 per cent N.							
451	89	89	8.0	101	23	85	23	461	82	90	8.0	108	10	46	77
452	96	95	8.0	101	21	84	24	462	82	92	8.0	108	9	44	78
453	92	87	8.0	101	23	84	28	463	80	88	8.0	108	10	48	74
454	94	92	8.0	101	22	89	20	464	83	96	8.0	108	8	38	82
455	112	113	7.8	99	21	80	29	465	87	101	8.0	108	9	39	79

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TABLE I—*Concluded.*

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.	Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
Period V. Egg ration, containing 0.65 per cent N.								Period V. Egg ration, containing 0.65 per cent N.							
	gm.	gm.	gm.	gm.	gm.	gm.	per cent		gm.	gm.	gm.	gm.	gm.	gm.	per cent
451	95	103	8 0		10	19		461	89	93	8 0		11	23	
452	99	109	7 8		11	17		462	91	96	8 0		11	24	
453	91	93	6 7		10	20		463	87	93	8 0		12	24	
454	93	98	6 3		8	16		464	97	103	8 0		12	20	
455	116	117	5 3		7	15		465	104	112	8 0		12	18	

butter fat, 10 per cent of sucrose, and enough starch to complete the ration. In one of the experimental periods these two rations were combined in the ratio of 3 parts of the tenderloin ration to 1 part of the cracklings ration. Each rat received per day, besides its allotment of ration (8.0 gm.), 25 mg.² of yeast vitamin (Harris), containing from 2 to 3 mg. of nitrogen, and 1 drop of cod liver oil.

In the standardizing periods (Nos. I and V) the rats received a ration similar to the above except that it contained a small amount of dried ether-extracted whole egg, the nitrogen of which is practically completely utilized in digestion and metabolism and hence would not be found to an appreciable extent in either the feces or the urine at this low level of feeding.

Ten albino rats, averaging 50 to 60 gm. in weight, were handled in two groups of five each by two persons. The order in which the three test rations were fed was reversed for the second as compared with the first group.

The results of the nitrogen balance studies (Table I) are used in the calculation of the biological value by a method fully explained elsewhere (7). The biological value obtained is taken as a measure of the percentage of the absorbed nitrogen that is used in the body for maintenance and growth under conditions in which maximal growth is restricted by the percentage of protein in the

² Except in Period II for Rats 461 to 465. In this period the vitamin allowance was increased to 50 mg. daily in the vain attempt to induce the rats to consume an adequate amount of food.

diet. The collection periods were of 7 days duration and were preceded by preliminary feeding periods of 4 days. A summary of the biological values is given in Table II.

The digestible nitrogen of pork tenderloin, containing an extremely low proportion of connective tissue, was found to possess a relatively high average biological value, *i.e.* 79, not much lower than that consistently found for milk under similar conditions, *i.e.* 84. The average biological value for the digestible nitrogen of pork cracklings was 25 for the first group of rats. The determination for the second group of rats cannot be considered satis-

TABLE II.
Summary of Biological Values.

Rat No.	Sex.	Pork tenderloin	Pork cracklings.	Mixture
451	F.	80	23	75
452	M.	81	24	69
453	"	80	28	68
454	F.	78	20	68
455	M.	78	20	68
461	F.	77	4	70
462	"	78	0	76
463	"	74	0	76
464	M.	82	0	76
465	"	79	0	74
Average.	79	25*	72

* Excluding the unsatisfactory determinations for Rats 461 to 465.

factory, because of the inadequate intake of food, ranging from 2.6 to 3.7 gm. per day. It is probable that the protein of pork cracklings is incomplete, since even with daily food intakes of 8 gm., the attainment of nitrogen equilibrium was impossible. This conclusion is in agreement with the results of the feeding experiments of Hoagland and Snider (8) on this material.

The biological values of the mixed ration, containing 3 parts of tenderloin nitrogen to 1 part of cracklings nitrogen, averaged 72. For Rats 451 to 455, in which the consumption of food was satisfactory in all periods, the mixed ration gave an average biological value of 70, while for the other group of rats, in which the con-

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sumption of food in the preceding (pork cracklings) period was inadequate, the average biological value was 74. Possibly the values for the mixed ration for this group of rats were raised somewhat above the correct value by the condition of inadequate nutrition prevailing throughout the preceding period of 11 days. For nine of the ten rats, the mixed ration gave a lower value than the tenderloin ration, so that the conclusion appears to be justified that the addition of connective tissue to the muscle tissue of the tenderloin cut had depressed the biological value.

There is good reason to suppose, however, that some supplementary relation exists between the nitrogenous compounds of muscle and connective tissue. Confining attention to the complete data for Rats 451 to 455, if no supplementary relation existed between the nitrogenous compounds of the two animal foods, the biological value of the mixture should have been

$$\frac{(79 \times 3) + 25}{4} = 65$$

Since the mixture actually showed a biological value of 70, some supplementary relation, by which the amino acid deficiencies of each product were corrected to some extent by the excess amino acid proportions of the other, was apparently at work.

The proportion of collagen + elastin nitrogen in the mixture of pork tenderloin and pork cracklings was 18.2 per cent of the total nitrogen. This is not as high as the percentages found in several cuts of veal analyzed in this laboratory. The fore shank of veal may run as high as 25 per cent of collagen and elastin nitrogen, so that for the mature animal a still higher per cent may be found. Furthermore, in both beef and pork the proportion of collagen to elastin nitrogen is always much greater than the proportion found in the pork cracklings used in this experiment (5). Therefore, if gelatin is lower in biological value than elastin, as appears probable, it would follow that a cut of meat containing 18.2 per cent of its total nitrogen in the form of collagen and elastin would possess a lower biological value than the mixture of tenderloin and cracklings used in this experiment. Hence, it appears possible to account for the variations noted above in the biological values obtained for different cuts of meat on the basis of a variable proportion of connective tissue.

It has been our experience that different cuts of pork possess rather constant biological values, averaging close to 74, in contrast to the variable values obtained for veal and beef. An explanation for this constancy is to be found in the comparatively constant amounts of connective tissue found in pork cuts. In Table III, the analyses for two hog carcasses are summarized, illustrating this fact.

Contrast with these series of values the variations in veal cuts ranging from 8 to 9 per cent of collagen N in the rib, round, sirloin, and tenderloin to 24 per cent in the fore shank, and from 0.5 per cent of elastin N in the tenderloin and rib to 4 or 5 per cent in the navel and the round.

TABLE III
Connective Tissue Content of Pork Cuts.

Results expressed as percentages of collagen or elastin N on the total N of the lean of the cut

	Ham		Loin		Picnic		Boston.		Belly.	
	Collagen	Elastin	Collagen	Elastin	Collagen	Elastin	Collagen	Elastin.	Collagen	Elastin.
Sow	7.8	1.0	8.5	0.8	11.3	1.0	8.0	0.6	7.9	0.6
Barrow	8.1	3.2	10.6	1.1	12.5	1.9	10.8	1.4	12.0	5.0

SUMMARY.

The biological value of the nitrogen of pork tenderloin, containing a minimal amount of connective tissue, was found to be 79. That of pork cracklings, consisting largely of connective tissue, was found to be 25.

When the two materials were mixed in the proportion of 3 parts of tenderloin nitrogen to 1 part of cracklings nitrogen, a distinct depression of the biological value of the tenderloin nitrogen was observed, the mixture possessing a value of 72.

Distinct indications of a supplementary relation between the nitrogenous compounds of muscle tissue and of connective tissue were noted.

Reasons are given for believing that cuts of beef varying widely in their content of connective tissue would also vary widely in the

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biological values of their nitrogen, so that the less desirable cuts, containing large amounts of connective tissue, would be distinctly less valuable as sources of protein for maintenance and growth. This is not so true of pork, since different cuts of pork do not seem to vary greatly in their content of connective tissue.

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